INVOLVEMENT OF MAST CELLS AND MAST CELL SERINE PROTEINASES IN EQUINE HEAVES

KIRSTIE JANE DACRE (nee PICKLES)

BVMS, MSc, Cert EM (Int Med), MRCVS

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DECLARATION

I declare that the contents of this thesis are my own work and that they have not been presented to any University other than the University of Edinburgh

Kirstie Jane Dacre

Edinburgh, January 2005

ABSTRACT

Mast cells release potent mediators upon degranulation, including serine proteinases. These proteinases play a pivotal role in the pathogenesis of human asthma. Due to the similarities between human asthma and equine heaves, a similar role for the mast cell in equine heaves is proposed.

Antibodies directed against equine tryptase and equine mast cell proteinase-1 (eq.MCP-1) were purified, validated and used to develop ELISAs for determination of proteinase concentrations in bronchoalveolar lavage fluid (BALF) from controls, clinical heaves horses, heaves horses in remission and horses with other pulmonary diseases. Clinical heaves horses had significantly increased BALF tryptase concentrations compared to controls or heaves horses in remission, whereas BALF tryptase concentrations of controls and heaves horses in remission did not significantly differ. Horses with other pulmonary diseases also had significantly elevated BALF tryptase concentrations compared to controls. Very low eq.MCP-1 concentrations were detected in all samples. Likewise, eq.MCP-1 immunoreactive cells were scarcely observed in equine lung tissue or BALF cytospins suggesting that eq.MCP-1 may be unimportant in the healthy and heaves affected equine lung.

Cloning and sequencing of these proteinases revealed an alanine 216 substitution in equine tryptase, which confers increased arginine substrate specificity and may restrict fibrinogenolysis *in vivo*. The deduced eq.MCP-1 Aa sequence differed from that isolated from equine mastocytoma tissue and it appears that a similar, but novel, chymase was sequenced. Probing of tryptase mRNA transcript regulation in control and heaves susceptible horses revealed no significant change in airway luminal cell pellet tryptase expression following hay/straw challenge of control or heaves horses. However, bronchiolar tissue from heaves horses in early resolution phase had significantly down-regulated tryptase transcripts compared to controls. Furthermore, immunohistochemistry revealed significant intra-epithelial recruitment of tryptase positive mast cells in heaves horses compared to controls, suggesting involvement of tissue mast cells in response to challenge.

In vitro hay dust suspension (HDS) challenge induced significant airway luminal mast cell degranulation in heaves susceptible horses, however a similar dose response trend was also evident in control horses. The increased number of intra-epithelial mast cells in heaves horses may explain the divergent mast cell response to *in vivo* and *in vitro* challenges. HDS-induced mast cell degranulation in both control and heaves horses may suggest non-IgE mediated degranulation. Alternatively, both control and heaves horses may have been sensitised to HDS allergens and phenotypic diversity may ultimately determine response to challenge. Collectively, these results provide evidence for mast cell involvement in the pathogenesis of equine heaves and warrant further studies into the potential roles of mast cell mediators.

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DEDICATION

To the memory of an inspirational friend,
Dean R. Prince (1965-2003).

The important thing is not to stop questioning.
Curiosity has its own reason for existing.
Albert Einstein

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Chapter 1: Review of the Literature

1.1. Introduction

As a result of domestication, horses are commonly exposed to deleterious stable environments that have long been associated with respiratory disease (Clarke, 1788). Consequently heaves (also termed recurrent airway obstruction, formerly chronic obstructive pulmonary disease), which is induced by inhalation of organic stable dusts, is the most common cause of chronic pulmonary disease in the horse in the United Kingdom (Dixon *et al.,* 1995a). Heaves is characterised by reversible neutrophilic airway inflammation and airway obstruction primarily due to bronchospasm and mucus hypersecretion following exposure of susceptible individuals to poor quality hay and straw (Robinson *et al.,* 1996) and as such, shares many similarities with human occupational dust induced asthma. Whilst great advances in knowledge have been made in recent years regarding the aetiological components of organic dusts that can produce clinical signs of equine heaves (McPherson *et al.,* 1979a; Halliwell *et al.,* 1993; McGorum *et al.,* 1993d; Schmallenbach *et al.,* 1998; Pirie, 2002); the pathogenesis of the pulmonary inflammatory response to inhaled dust is still poorly understood. Similarly, due to the characteristic neutrophil influx into the pulmonary airspace following challenge of heaves susceptible horses, it is the neutrophil that has been the focus of most research, whilst the cellular events orchestrating this neutrophilic response have received comparatively little attention.

The cell population of the healthy airway comprises macrophages, lymphocytes and occasional mast cells, neutrophils and eosinophils. The mast cell has tremendous ability to react rapidly to inhaled stimuli releasing many potent mediators, including serine proteinases, biologic amines and cytokines, with potential to influence a wide range of effector cells. These mediators are believed to be biologically important in the lung (Caughey, 1991) with mast cell degranulation acting as a key event in

initiating and maintaining airway response to challenge in human asthma (Holgate, 2000). By analogy, a similar role for the mast cell may exist in equine heaves.

Current treatment protocols for heaves include preventative measures, such as environmental control to remove the inciting dusts (which is often impractical in temperate climates), or symptomatic therapy such as anti-inflammatories and bronchodilators. It has become increasingly evident over recent years that the clinical features of heaves are consequences of the underlying pulmonary inflammatory response. Effective management of heaves therefore centres upon a fuller understanding of the inflammatory pathway to allow more rational, directed therapies for the treatment of this disease.

1.2. Equine Heaves

1.2.1. Aetiology

Although agents have been recognised that produce the clinical signs of heaves, the definitive aetiologic agents or mechanisms that induce disease are not yet defined. It is likely that heaves is multifactorial in aetiology, however the most commonly implicated factor is exposure of the horse to environmental respiratory allergens, predominantly in the stable environment (McPherson *et al.,* 1979a; McPherson *et al.,* 1979b; Derksen *et al.,* 1985; Tremblay *et al.,* 1993; McGorum *et al.,* 1993d). Although the term 'respiratory allergen' has been used in many studies, the role of allergy in equine heaves is still equivocal and poorly understood, as discussed furthered in section 1.2.4.1.

1.2.1.1. Organic Stable Dust

Horses are exposed to a myriad of airborne pollutants in stable dust which can act as allergens or direct irritants to the lung. These agents include inorganic dusts, fungal and actinomycete spores, endotoxin, bacteria and viral aerosols, noxious gases such as ammonia, plant material and dust mites and their metabolites (Clarke, 1987; McGorum *et al.,* 1998; May and Robinson, 2004). A genetic predisposition of susceptibility to these environmental dusts has been described (Marti *et al.,* 1991).

The primary sources of airborne stable dusts are hay and straw and microbial growth *in situ* on deep litter bedding materials such as wood shavings or newspaper (McPherson *et al.,* 1979b; Clarke, 1987; Spendlove *et al.,* 2004). Agitation of food and bedding due to foraging behaviour by the horse results in the dust concentrations around the muzzle ('the breathing zone') being much higher than that in the overall stable environment (Woods *et al.,* 1993). Some degree of fungal contamination is present in all hays. However, severe fungal growth occurs in hay that is baled with a high moisture content, which can result in respirable challenges of up to 10^{10} fungal and actinomycete propagules per breath in the horses' breathing zone (Clarke, 1987).

Dust concentrations in the stable vary with the feed and bedding products used (Woods *et al.,* 1993; Vandenput *et al.,* 1997; McGorum *et al.,* 1998; Spendlove *et al.,* 2004). A conventional stable with straw and visibly mouldy hay may have breathing zone dust concentrations as high as 17.5mg/m^3 total dust and 9.3mg/m^3 respirable dust (Woods *et al.,* 1993). In contrast, the current recommended threshold limiting value for humans with an average 8h exposure period to grain dust is 4mg/m^3 total dust (Chan-Yeung *et al.,* 1992) and grain workers with repeated 8h exposure to total dust concentrations of 5mg/m^3 have suffered serious loss of pulmonary function (Enarson *et al.,* 1985). Considering that permanently housed horses may be almost continuously exposed to the aforementioned much higher levels of dust, ensuing airway dysfunction would seem highly probable in these horses.

Clinical remission of heaves can be achieved by reduction of the environmental dust burden, optimally by turning the horse out to pasture (McPherson *et al.,* 1978; Derksen *et al.,* 1985). Keeping horses permanently outdoors however is frequently impossible or impractical for owners in cold or temperate climates and therefore environmental control is often instigated by improving stable ventilation and the use of low dust feed substitutes such as haylage, haymax, silage or complete pelleted diet and of beddings such as paper, shavings or rubber mats. Low dust stable environments using wood shavings bedding and feeding a pelleted diet contain up to 97% less respirable dust burden in the equine breathing zone compared to that in

conventional, poorly ventilated stables containing hay and straw (Woods *et al.,* 1993; McGorum *et al.,* 1998). However, horses in low dust management stables have significantly higher dust concentrations in the breathing zone than those at pasture (McGorum *et al.,* 1998). As mould spores are a large component of stable dust, concentrations of these spores are also significantly lower in a low dust stable environment (Woods *et al.,* 1993). Vandenput *et al.* (1998) were able to maintain heaves horses in clinical remission in a well ventilated stable bedded with good quality straw and by feeding grass silage. However, when silage was replaced by good quality hay, deterioration of pulmonary function was evident after several days suggesting that, at least in this study, the hay was more capable of producing clinical signs of heaves than the straw.

1.2.1.2. Mould Spores

Potential allergens that have frequently been implicated in the aetiology of equine heaves include the mould spores *Faenia rectivirgula*, *Aspergillus fumigatus* and *Thermoactinomyces vulgaris* (McPherson *et al.,* 1979a; Derksen *et al.,* 1988; Woods *et al.,* 1993; McGorum *et al.,* 1993d). These mould spores are respirable, i.e. have an aerodynamic diameter <5μm and as such are small enough to be inhaled into the terminal airways. Inhalation challenge of heaves susceptible horses with *Faenia rectivirgula* and *Aspergillus fumigatus* extracts have been reported to result in increased maximum trans-pulmonary pressure change (ΔPpl), decreased arterial oxygen pressure (PaO_2) (McPherson *et al.,* 1979a), deterioration in pulmonary mechanics (Derksen *et al.,* 1988) and intraluminal neutrophil recruitment (McGorum *et al.,* 1993d), consistent with a heaves-like response. Although Derksen *et al.* (1988) found that inhalation challenge with *Faenia rectivirgula* increased bronchoalveolar lavage fluid (BALF) neutrophil counts in both control and heaves susceptible horses, the high challenge concentrations of antigen used were within the ranges which have induced false positive responses in humans with no history of respiratory disease (Cavannagh *et al.*, 1977). *Aspergillus fumigatus* and *Faenia rectivirgula* are further implicated by the observation that heaves susceptible horses have increased levels of BALF IgE to these agents compared to control horses (Halliwell *et al.,* 1993; Schmallenbach *et al.,* 1998). The role of *Thermoactinomyces vulgaris* is less clear, as

inhalation studies with this potential allergen caused a neutrophilic influx into the airway lumen in both control and heaves susceptible horses (McGorum *et al.,* 1993d). Inhalation challenge with these three mould extracts induced less severe intraluminal neutrophilic influx than following natural hay / straw exposure and no significant deterioration in lung function occurred following challenges (McGorum *et al.,* 1993d). This suggests that the duration of exposure to these fungal extracts, or inhalation of a combination of antigens and pro-inflammatory agents is likely to be important in reproducing clinical heaves.

On reflection of more recent literature however, it is possible that response to these fungal extracts may have been at least partly due to non-specific responses to contaminant endotoxin, rather than the fungal antigens themselves (Pirie, 2002). Furthermore, the former studies utilised only soluble fungal antigens and the presence of combined soluble antigens and particulate matter have proven important in simulating the features of natural challenge (Pirie, 2002).

1.2.1.3. Storage Mites

Large numbers of storage mites have been found in poorly saved forage and are clinically important allergens in some forms of occupational asthma (Hallas and Gudmundsson, 1985; Solarz *et al.,* 1997). However, heaves horses do not have elevated BALF IgE or IgG specific for these mites *(B.C. McGorum, personal communication)* suggesting that hypersensitivity to these mites is unlikely to play a role in the heaves response.

1.2.1.4. Endotoxin

Airborne endotoxin concentrations correlate with environmental dust concentrations and have recently been implicated as an auxiliary aetiological agent causing pulmonary inflammation in heaves (McGorum *et al.,* 1998; Pirie *et al.,* 2001; Pirie, 2002). Certainly, conventional hay / straw stables have been shown to contain endotoxin concentrations in excess of those which can induce bronchial hyper-responsiveness and pulmonary inflammation in control human subjects and bronchoconstriction in humans with pre-existing pulmonary inflammation

(McGorum *et al.,* 1998). Inhalation challenge with endotoxin induces a dose dependent neutrophilic airway inflammatory response in both heaves susceptible and control horses; however heaves susceptible horses have a lower response threshold (Pirie *et al.,* 2001). Lung dysfunction was only observed in heaves susceptible horses and only following higher challenge doses (Pirie *et al.,* 2001). Comparison of natural hay / straw exposure and nebulised endotoxin challenges suggests that whilst inhaled endotoxin may contribute to airway inflammation, or even act synergistically with other dust components, it is not the sole cause of the clinical signs in heaves affected horses when exposed to hay / straw (Pirie *et al.,* 2001).

1.2.2. Pathology

The main pathological finding in equine heaves is bronchiolitis (Nicholls, 1978; Winder and von Fellenberg, 1988; Beech, 1991) characterised by diffuse epithelial hyperplasia, increased numbers of goblet cells and goblet cell metaplasia, decreased numbers of non-ciliated (Clara) cells, mucus plugging of airways and peribronchiolar infiltration of neutrophils, lymphocytes and plasma cells (Thurlbeck and Lowell, 1964; Nicholls, 1978; Winder and von Fellenberg, 1988). In more severely affected chronic cases, peribronchiolar fibrosis and alveolar emphysema may result (Winder and von Fellenberg, 1988; Kaup *et al.,* 1990a; Derksen, 1991). Eosinophilic infiltration may be present in occasional animals (Thurlbeck and Lowell, 1964).

Despite similarities between equine heaves and human asthma, these diseases differ in the predominant inflammatory cell recruited into the airway lumen. Thus, whilst heaves is characterised by neutrophil influx into the airway lumen following challenge of heaves susceptible horses (Winder and von Fellenberg, 1988; Dixon *et al.,* 1995b; Freeman and Roszel, 1997), classical asthma is characterised by recruitment of predominantly eosinophils (Coyle *et al.,* 1996). Heaves is therefore more analogous to human occupational dust induced asthma, which also has a predominantly neutrophilic cellular influx into the airway lumen. The mechanism for inducing this neutrophilic rather than eosinophilic influx is not definitively known but has been suggested to result from the key transcription factor nuclear factor-κB

(NF-κB) in bronchial epithelial and airway luminal cells from heaves affected horses being composed mainly of p65 homodimers, rather than classical p65-p50 heterodimers (Bureau *et al.,* 2000). These p65 homodimers induce expression of intercellular adhesion molecule-1 (ICAM-1), a cell surface ligand required for neutrophil migration (Ledebur and Parks, 1995), and the potent neutrophil chemokine IL-8 (Schulte *et al.,* 2000), in contrast to the p65-p50 heterodimers which induce expression of the eosinophil chemoattractant eotaxin (Matsukura *et al.,* 1999). However, mast cell mediators could provide an alternative explanation for this neutrophil recruitment as mast cell derived tryptase, tumour necrosis factor-α (TNF-α), interleukin-4 (IL-4), IL-8 and extractable nuclear antigen-78 (ENA-78) are all potent chemokines with numerous other additional effects on mechanisms of neutrophil recruitment as discussed in section 1.4. In addition, grain dust has been demonstrated to induce both direct and indirect neutrophil chemotaxis via several different pathways (section 1.3.1) and therefore in a similar mechanism, it is possible that organic stable dust also plays a role in neutrophil recruitment to the airway.

Ultrastructural studies of the larger airways confirm that the principal change in heaves affected horses is a hyperplastic epithelium with focal loss of ciliated epithelial cells replaced by undifferentiated cells (Kaup *et al.,* 1990b). Dilated intercellular clefts and accumulations of mast cells, considered to be indicative of non-specific mucosal hyper-reactivity, are also present (Kaup *et al.,* 1990b). There are degenerative changes in the terminal airways, including lack of differentiation and loss of granulation of Clara cells and metaplasia of goblet cells. Alveolar regions show necrosis of type I epithelial cells, alveolar fibrosis of varying degrees and type II epithelial transformation and emphysema with an increase in Kohn's pores (Kaup *et al.,* 1990a).

The pathological lesions of heaves, although of a diffuse nature, are not homogenous in distribution (Derksen, 1991). Whilst cytological composition of BALF samples collected from 4 different lung regions in heaves affected horses showed no significant difference (McGorum *et al.,* 1993e), inflammatory markers indicative of

oxidative stress and oxidative damage may show regional differences (*D. Marlin, personal communication*).

1.2.3. Pathophysiology

Heaves is characterised by reversible small airway obstruction, airway hyper-reactivity and inflammation in response to inhalation of organic dusts. Airway obstruction and subsequent deterioration of pulmonary function is predominantly the result of bronchospasm of the bronchiolar smooth muscle, largely due to activation of M3 muscarinic receptors by acetylcholine (Broadstone *et al.,* 1988; Robinson *et al.,* 1996). The *in vitro* response of airway smooth muscle to acetylcholine is unaltered, and therefore this increased smooth muscle tone is thought to be due to activation of airway reflexes by inflammatory mediators, decreases in inhibitory mechanisms such as the intrapulmonary nonadrenergic noncholinergic nervous system and the production of PGE_2 in affected horses (Robinson *et al.,* 1996). Pulmonary function indices of heaves affected horses at peak bronchodilator response are significantly impaired relative to those of control horses suggesting that residual obstruction is due to peribronchiolar cellular infiltration and luminal mucus accumulation (Thurlbeck and Lowell, 1964; Murphy *et al.,* 1980).

Diffuse airway obstruction leads to ventilation / perfusion mismatching and a normocapnic hypoxaemia (McPherson and Thomson, 1983). The increased respiratory drive caused by hypoxaemia in the presence of airway obstruction leads to affected horses adopting a characteristic breathing pattern in which very high peak flows at the start of exhalation rapidly diminish as expiration proceeds (Robinson *et al.,* 1996). With severe disease, horses develop a pronounced abdominal end expiratory effort; the so-called 'heave'. Heaves susceptible horses exhibit reduced dynamic compliance and increased pulmonary resistance, which increase the work of breathing and ΔPpl, following exposure to natural challenge environments (Muyulle and Oyaert, 1973; McPherson *et al.,* 1978; Thomson and McPherson, 1984; McGorum *et al.,* 1993d; Vandenput *et al.,* 1998). Respiratory rate, minute volume, maximum inspiratory flow rate and ratio of expiratory to inspiratory time are also

significantly increased whilst inspiratory time and PaO_2 decrease in symptomatic heaves horses (Muyulle and Oyaert, 1973; Thomson and McPherson, 1984).

Intraluminal airway cytology, airway reactivity and pulmonary function values return to normal following removal of inhalational challenge. Remission of clinical signs occurs 8.4 ± 4.8d (mean ± s.d., range 4 - 24d) following implementation of environmental control (Thomson and McPherson, 1983; Thomson and McPherson, 1984; Armstrong *et al.,* 1986; McGorum *et al.,* 1993d). The time taken for horses to become asymptomatic has been correlated with age, duration of illness and severity of disease, as judged by the non-elastic work of breathing (Thomson and McPherson, 1984). Some structural airway changes such as epithelial metaplasia and hyperplasia and smooth muscle hyperplasia take longer to resolve (Robinson *et al.,* 1996). Excessive and disordered mucus secretion and / or delayed mucokinesis may also persist for prolonged periods following removal of allergens from the environment (Dixon *et al.,* 1995b).

1.2.4. Pathogenesis

Despite much research, the specific pathways which result in pulmonary inflammation and obstruction in equine heaves remain ill-defined. In particular, the role of allergy in the pathogenesis still remains somewhat speculative.

Collectively, the presented evidence suggests involvement of an IgE-mediated hypersensitivity, but that it is unlikely to be the sole mechanism responsible for the equine heaves response. Phenotypic diversity of heaves affected horses, as has been described for occupational human asthmatics, is suggested by the individual variability of response to challenge and the contrasting reports of IgE and T-helper cell 2 (Th2) responses in affected horses. Consequently, phenotypic variation may be an important determinant of the individual horse's response to inhalational challenge.

1.2.4.1. Hypersensitivity to Moulds and Other Allergens

There is credible evidence from inhalational challenge studies to support the role of hypersensitivity to the previously mentioned mould species in the pathogenesis of heaves. Challenge with *Faeni rectivirgula* and *Aspergillus fumigatus* extracts resulted in intraluminal neutrophilic influx in heaves susceptible but not control horses, suggestive of a pulmonary hypersensitivity to specific antigens rather than a non-specific response to dusts and irritants in the stable environment (McGorum *et al.,* 1993d). Increased levels of BALF IgE antibodies to *Faeni rectivirgula* and *Aspergillus fumigatus* in asymptomatic and symptomatic heaves susceptible horses also support a type I (IgE mediated) hypersensitivity response (Halliwell *et al.,* 1993; Schmallenbach *et al.,* 1998). These studies reported IgE involvement to be restricted to a localised pulmonary response as systemic antigen specific IgE levels were not elevated in affected animals. More recent evidence however, suggests that serum IgE to grain dust and grass pollens is in fact increased in a significant proportion of heaves susceptible horses (*L. Monreal, personal communication*). An increase in IgE positive cells in bronchiolar tissue and pulmonary blood vessels has also been found in heaves affected horses as compared to controls, although this effect was predominantly caused by very high numbers of positive cells found in two of the five heaves horses (van der Haegen *et al.,* 2001).

The selective recruitment of CD4+ T (helper) lymphocytes from peripheral blood into the airway lumen following natural hay / straw challenge of heaves susceptible horses also supports the role of allergy in the pathogenesis of heaves (McGorum *et al.,* 1993b). Similarly, the cytokine profile of increased IL-4 and IL-5 and decreased interferon-γ (INF- γ) expression in airway luminal cells from heaves affected horses as compared to controls is consistent with a local pulmonary Th2 response (Lavoie *et al.,* 2001). Other cytokine studies however, have suggested that heaves is not characterised by a Th2 response (Giguere *et al.,* 2002; Ainsworth *et al.,* 2003b).

As airway obstruction and intraluminal neutrophil accumulation occur several hours after antigen exposure, the response is most typical of a late phase, rather than an immediate phase, type I hypersensitivity (McGorum *et al.,* 1993d; Robinson *et al.,*

1996). Involvement of a late phase response is also suggested by elevated pulmonary epithelial lining fluid (PELF) histamine concentrations in heaves susceptible ponies at 5h, but not at 0.5h, post natural challenge (McGorum *et al.,* 1993c). However, histamine release from mast cells is not necessarily IgE mediated as discussed further in section 1.2.4.2.

Halliwell *et al.* (1979) previously suggested that IgE (type I), IgG (type III) and possibly cell mediated (type IV) hypersensitivities were involved in the pathogenesis of heaves. However that study was based upon skin wheal response to intradermal antigens which, subsequent studies have shown do not correlate with pulmonary responses to mould extracts (McGorum *et al.,* 1993a).

1.2.4.2. Evidence for a Mast Cell Role

The aforementioned studies demonstrating evidence of IgE mediated type I hypersensitivity support mast cell participation in the pathogenesis of heaves. Increased histamine release following *in vitro Aspergillus fumigatus* challenge of airway luminal cells from symptomatic and asymptomatic heaves horses compared to controls suggests that mast cells from heaves susceptible horses may be sensitised to these fungal allergens (Hare *et al.,* 1999). Furthermore, *in vivo* hay / straw challenge caused significantly elevated PELF histamine concentrations in heaves susceptible horses but not in controls, with concentrations correlated to numbers of metachromatically stained intraluminal cells (McGorum *et al.,* 1993c).

Histological and ultrastructural studies have demonstrated large numbers of mast cells in the normal equine lung, with approximately 20% of these present in the bronchiolar walls, the primary site of histological change in horses with heaves (Mair *et al.,* 1988). Mast cells are also located in the airway epithelium (Mair *et al.,* 1988) and the airway and alveolar lumina where they are strategically placed to interact with inhaled antigens (Kaup *et al.,* 1990a). Increased numbers of peribronchiolar mast cells are reported in horses with mild, moderate and severe chronic bronchiolitis (Nicholls, 1978; Winder and von Fellenberg, 1990). Furthermore, ultrastructural studies have shown intraepithelial accumulations of mast cells in heaves affected

horses in varying stages of degranulation (Kaup *et al.,* 1990b). Degranulated mast cells have also been identified in normal human bronchial mucosa however, and therefore it is likely that some degranulation is physiological, rather than pathological in nature (Lamb and Lumsden, 1982).

Some studies have also found increased numbers of intraluminal mast cells in symptomatic heaves horses (Yashamiro *et al.,* 1986; Winder *et al.,* 1990; Vrins *et al.,* 1991) or horses with airway hyper-reactivity (Hoffman *et al.,* 1998). Indeed, in this latter study, intraluminal mast cell counts were significantly correlated with airway hyper-reactivity. Degranulated mast cells have also been observed in BALF preparations from horses with heaves (Yashamiro *et al.,* 1986; Vrins *et al.,* 1991) suggesting release of mast cell mediators may occur in the airway lumen. Mast cell granules contain mediators capable of causing many of the pathophysiological effects of heaves as discussed in section 1.4. Indeed, Olszewski *et al.* (1999) demonstrated that mast cell mediators increase cholinergic small airway tone *in vitro* suggesting a potential role for these agents in the development of bronchospasm and airway obstruction (Olszewski *et al.,* 1999).

The apparent effectiveness of sodium cromoglycate, which is reputed to have mast cell stabilising effects, in the prophylaxis of heaves may provide further evidence for a mast cell role (Murphy *et al.,* 1979; Thomson and McPherson, 1981). However, studies in man have shown that the beneficial effects of this drug are more likely to be attributable to its other anti-inflammatory effects (Richards *et al.,* 1986).

Although mast cell participation in the pathogenesis of equine heaves is consistent with a type I hypersensitivity response, mast cell degranulation is not necessarily IgE mediated. It is now recognised that mast cells may also degranulate following non-immunological activation by complement, neuropeptides, cytokines, endotoxin, adenosine, lectins, opioids, hypoxia and changes in osmolality (da Silva *et al.,* 1967; Church *et al.,* 1989; White, 1990; Bingham and Austen, 2000; Crummy *et al.,* 2004).

1.2.4.3. Previous Viral Injury

Several studies have shown that a significant proportion of heaves horses (19.2-65.8%) have a history of respiratory infection in the immediate time period prior to onset of heaves (McPherson *et al.,* 1978; Halliwell *et al.,* 1993; Dixon *et al.,* 1995a). Thorsen *et al.* (1983) found increased levels of anti-haemagglutination antibodies in respiratory secretions from horses with heaves as compared to controls. Increased levels of BALF IgE for *Faeni rectivirgula* and *Aspergillus fumigatus* have also been identified in horses with a prior history consistent with respiratory viral infection and which continued to cough after the expected resolution of the infectious disease (Halliwell *et al.,* 1993). Furthermore, inflammatory airway disease in young horses, in which respiratory infections may have an aetiological role, has been hypothesised to progress into heaves in the older animal (Moore *et al.,* 1995; Hare and Viel, 1998) Although the specific method by which viral or bacterial lung disease acts as a trigger for the induction of heaves is unknown, it appears that prior infection may increase the subsequent immune response to potential antigens.

1.3. Occupational Dust Induced Asthma

Occupational dust induced asthma (hereafter termed occupational asthma) is characterised by reversible bronchiolitis and airway obstruction, as a result of inhalation of environmental dusts in the workplace. In contrast to the intraluminal influx of eosinophils in classical asthma, occupational asthma is characterised by neutrophilic recruitment into the pulmonary airspace (Von Essen *et al.,* 1988; Park *et al.,* 1999). Occupational asthma therefore shares many similarities with equine heaves, which itself has been described as an occupational lung disease as it is a consequence of the environment in which horses are kept in order to perform (Derksen, 1993).

Human occupational asthma has been reported in work environments associated with chronic inhalation of many types of organic dusts including grain dust (Do Pico *et al.,* 1984; Chan-Yeung *et al.,* 1992; Park *et al.,* 1999), red cedar sawmill dust (Vedal *et al.,* 1986; Frew *et al.,* 1993; Chan-Yeung, 1994), cotton (Schachter *et al.,* 1984;

Zuskin *et al.,* 1997), toluene diisocyanate (Di Stefano *et al.,* 1993), storage mites (Alvarez *et al.,* 1999), vegetable gums (Bush, 1990) and fish processing (Rodriguez *et al.,* 1997). These multiple subtypes of occupational asthma do not appear to share a universal pathogenesis for induction of pulmonary inflammation. Therefore, due to the aetiological similarities between grain dust induced human occupational asthma and equine heaves, this review will focus upon literature regarding grain dust associated occupational asthma. Although the interstitial lung disease extrinsic allergic alveolitis (Farmer's Lung) occurs in humans exposed to mouldy hay and straw, this is a hypersensitivity pneumonitis (involving the alveoli and pulmonary interstitium) rather than a type of occupational asthma (involving airways) and therefore will not be reviewed.

1.3.1. Grain Dust Associated Occupational Asthma

The composition of grain dust is variable but is predominantly composed of fractured grain kernels, fractured weed seeds, husks, storage mites, insects, bacteria, moulds, inorganic matter and chemicals (Chan-Yeung *et al.,* 1992). The effects of grain dust inhalation have long been associated with respiratory ill health, with up to 88% of grain workers reported to experience respiratory symptoms in the 1970's (Do Pico *et al.,* 1977). This high disease prevalence has lead to much research in recent decades, with progressive reductions in the recommended threshold limiting value for environmental dust exposure (currently 4mg/m^3 total grain dust for an average 8h exposure period) (Chan-Yeung *et al.,* 1992).

The prevalence of respiratory symptoms among grain workers is positively correlated with length of employment in the grain industry (Do Pico *et al.,* 1984). Predisposing host factors such as atopy and smoking increase the severity of disease and are important determinants of the individual's response to grain dust exposure (Do Pico *et al.,* 1984; Chan-Yeung *et al.,* 1992; Park *et al.,* 1998a). Skin reactivity to common allergens is not associated with this disease (Enarson *et al.,* 1985; Park *et al.,* 1998a). Grain dust specific IgE is present in the serum of 40% of symptomatic subjects compared to only 11% of asymptomatic subjects (Park *et al.,* 1998a)

suggesting that grain dust can induce a systemic IgE-mediated response in some individuals. However, as the majority of sufferers do not have serum specific IgE, other non-IgE-mediated responses are also likely to be involved in the pathogenesis of grain dust occupational asthma.

A strong dose-response relationship exists between grain dust concentration and both respiratory symptoms and physiological lung dysfunction (Enarson *et al.,* 1985; Huy *et al.,* 1991) with chronic exposure to $5mg/m^3$ total grain dust resulting in a serious reduction in lung function (Enarson *et al.,* 1985). Wheat grain extract causes a direct, dose dependent constriction of guinea pig tracheal smooth muscle *in vitro,* possibly by activation of cholinergic receptors (Schachter *et al.,* 2004). Direct, dose-dependent histamine release from human lung mast cells occurs following *in vitro* grain dust challenge (Chan-Yeung *et al.,* 1987; Alam *et al.,* 1988) suggesting mast cell involvement in the induction of this pulmonary dysfunction. This direct histamine release has been shown to occur independently of IgE, complement and cell cytotoxicity (Chan-Yeung *et al.,* 1987). Grain dust also induces indirect histamine release by stimulating the production of a histamine releasing factor from lymphocytes (Alam *et al.,* 1988). This lymphocyte derived degranulating factor is suggested to be of greater physiological significance *in vivo* as its release occurs with much lower grain dust concentrations than are required to induce direct mast cell degranulation (Alam *et al.,* 1988).

Grain dust is capable of inducing both direct and indirect neutrophil chemotaxis *in vitro,* which may be important mechanisms for neutrophil recruitment into the airway lumen *in vivo*. Aqueous extracts of grain dust are capable of activating both classical and alternative complement pathways (Olenchock *et al.,* 1980) such that indirect chemotaxis occurs via release of the potent neutrophil chemotactic factor C5a (Von Essen *et al.,* 1988). Grain dust also induces the release of a neutrophil chemoattractant factor from both bronchial epithelial cells (Von Essen *et al.,* 1994) and alveolar macrophages (Von Essen *et al.,* 1988). IL-8 has been suggested as the inducible neutrophil chemokine and its concentration is significantly increased from baseline in the sputum of occupational asthmatics following *in vivo* grain dust

challenge (Park *et al.,* 1998b). Increased serum neutrophil chemotactic activity is also evident in occupational asthmatics 30min following grain dust challenge (Park *et al.,* 1999). This serum neutrophil chemotactic activity can be divided into heat stable and heat labile factors (Atkins *et al.,* 1977) which are believed to originate from mast cells and monocytes / macrophages, respectively (Nagy *et al.,* 1982). The numbers of mast cells in the bronchial mucosa are significantly increased in grain dust occupational asthmatics compared to allergic asthma sufferers (Park *et al.,* 1998b) further supporting their involvement in pathogenesis of this disease.

1.4. Mast Cells in Airway Disease

1.4.1. Mast Cell Activation

Mast cells are strategically located close to blood vessels and nerves in tissues that interface with the external environment, for example the lung, to encounter and respond to foreign antigens. Mast cell activation is traditionally described as the result of crosslinkage of allergen specific IgE to high affinity FcεRI receptors on the mast cell surface (Kinet, 1990). However, as previously mentioned, degranulation can also be mediated by endotoxin, complement, neuropeptides, adenosine, opioids, hypoxia, changes in osmolality and lectins (Eggleston *et al.,* 1987; Church *et al.,* 1989; White, 1990; Heaney *et al.,* 1995; Bingham and Austen, 2000; Polosa *et al.,* 2002). Therefore allergy is not a prerequisite for mast cell activation. Following activation via immunoglobulin receptors, polypeptide ligands (Bingham and Austen, 2000) or c-kit receptors (Bischoff and Dahinden, 1992) mast cells exocytose preformed mediators stored in secretory granules such as the proteinases tryptase and chymase and the biologic amine histamine. Activated mast cells also synthesise substantial quantities of newly formed lipid mediators, such as leukotrienes (LT) and prostaglandins (PG), and express multi-functional cytokines (Church *et al.,* 1989; Bingham and Austen, 2000). These mediators have a wide range of effector cells and in asthma are believed to play a role in increasing vascular permeability and causing bronchoconstriction, mucus hypersecretion and cellular recruitment, to amplify the inflammatory response and ultimately contribute to tissue remodelling (Bingham and

Austen, 2000). Mast cell mediators and their target cells are shown schematically in fig. 1.1.

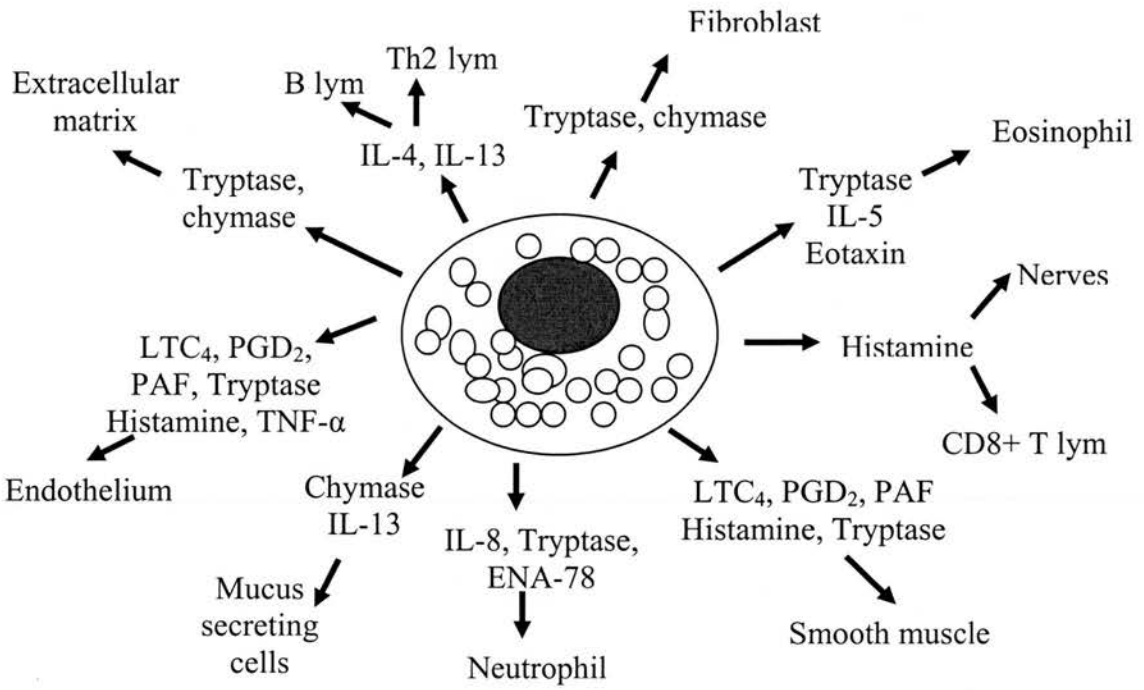

Fig.1.1: Schematic diagram to show the integrated role of mast cell products in the development of allergic airway inflammation. lym = lymphocyte, IL = interleukin, LTC = leukotriene C; PGD = prostaglandin D; PAF = platelet activating factor; ENA = extractable nuclear antigen, TNF-α = tumour necrosis factor-α. Taken from Hart (2001).

1.4.2. Mast Cell Heterogeneity

Mast cells can be defined by their tissue location into mucosal mast cells (MMC) and connective tissue type mast cells (CTMC). They have also been classified according to their proteinase content into mast cells containing only tryptase (MC_T), mast cells containing both tryptase and chymase (MC_{TC}) and mast cells containing only chymase (MC_C). Mast cell heterogeneity has been demonstrated in many species with regard to their proteinase and cytokine content and also their function, with these being dependent upon their tissue location (Bienenstock *et al.,* 1985; Irani *et al.,* 1986; Barrett and Metcalfe, 1987; Shanahan *et al.,* 1987; Chen *et al.,* 1990; Sture *et al.,* 1995; Kube *et al.,* 1998; Kuther *et al.,* 1998). In rodents, mast cell

heterogeneity is particularly notable with regard to their chymase content. For example, the chymase mouse mast cell proteinase-4 (mMCP-4) is found in CTMC and chymases mMCP-1 and mMCP-2 are found in MMC (Bienenstock *et al.,* 1985; Barrett and Metcalfe, 1987). However, there is also mast cell heterogeneity among species such that mast cell proteinase composition in particular tissue locations differs with the species studied. For example, in humans, MC_T are found in the lung and intestinal mucosa whilst MC_{TC} are characteristically found in connective tissue locations such as skin, lymph nodes and intestinal submucosa and occasional MC_C are found in human lung and intestinal tissue (Irani *et al.,* 1986; Weidner and Austen, 1990; Weidner and Austen, 1991). In contrast, the majority of sheep mast cells are MC_T however gastrointestinal and lung nematodes induce a chymase (sheep mast cell proteinase-1 [SMCP-1]) containing mast cell population. Parenchymal mast cells are then MC_{TC} but airway MMCs are predominantly MC_T (*J. Brown, personal communication*). A preliminary study of the tissue distribution and heterogeneity of equine mast cells reported the presence of chymase containing cells in connective tissue and MC_T in both mucosal and connective tissue locations (Pemberton *et al.,* 2001). Chymases are divided into α- and β- chymases according to their ability or inability respectively to catalyse the conversion of angiotensin I to angiotensin II (Wintroub *et al.,* 1984; Chandrasekharan *et al.,* 1996). Homologous α-chymases are expressed in human and canine mast cells whereas rodent and ruminant mast cells can contain both α- and β-chymases (Miller and Pemberton, 2002). It may be inappropriate to compare chymase mast cell heterogeneity between species expressing α- and β-chymases, which could have differing tissue distributions and functions.

The characteristic metachromatic staining of mast cell granules seen with toluidine blue is due to their content of heavily sulphated anionic proteoglycans, heparin and chondroitin sulphate glycosaminoglycan, which act to stabilise proteinases in the granule (Bingham and Austen, 2000). CTMC synthesise heparin whereas MMC of most species produce little or no heparin and instead synthesise lower sulphated chondroitin sulphates. MMC do not stain well with toluidine blue following aldehyde fixation, although the reason for this is not clear (Walls *et al.,* 1990).

1.4.3. Evidence for Mast Cell Involvement in Human Asthma

It has been increasingly recognised over recent years that the release of mast cell mediators may play a central role in inflammatory disorders such as allergic and occupational asthma (Kaliner, 1984; Marone, 1985; Kay, 1987; Hart, 2001). As previously mentioned, mast cell density in the lung is highest around blood vessels, nerves and mucus glands; key areas where they are able to effect a rapid response to a noxious insult. Allergen challenge in sensitised asthmatics induces both mast cell infiltration into the airway mucosa and subsequent degranulation (Casale *et al.,* 1987b; Pesci *et al.,* 1993; Montefort *et al.,* 1994). Similarly, intra-epithelial accumulations of mast cells have been observed in the airways of heaves affected horses (Kaup *et al.,* 1990b), inviting speculation of a similar mast cell response to challenge in equine heaves and human asthma.

Compared to control subjects, asthmatics have increased numbers of mast cells in their sputum (Pizzichini *et al.,* 1996) and bronchial mucosa (Lozewicz *et al.,* 1988; Crimi *et al.,* 1991; Pesci *et al.,* 1993; Gibson *et al.,* 1993; Laitinen *et al.,* 1993; Bradding *et al.,* 1994). These bronchial mucosal mast cells demonstrate progressive degranulation towards the airway lumen (Pesci *et al.,* 1993; Koshino *et al.,* 1995). Increased numbers of intraluminal mast cells are inconsistently reported in asthmatics, however if present, a positive correlation with airway hyper-reactivity may exist (Kirby *et al.,* 1987). The number of mast cells in asthmatic airway epithelium have also been reported to correlate with the number of intraluminal mast cells and airway reactivity (Gibson *et al.,* 1993).

Mast cell numbers are also increased in bronchial smooth muscle of human asthmatics compared to controls, suggesting they may play a role in bronchial hyper-responsiveness (Ammit *et al.,* 1997; Brightling *et al.,* 2002; Carroll *et al.,* 2002a). Indeed, passive sensitisation of human bronchi with asthmatic serum *in vitro* induced mast cell degranulation with an associated increase in measurable tryptase and airway hyper-responsiveness (Berger *et al.,* 2002). Mast cells appear essential to the

development of this hyper-reactive airway as previously sensitised control mice and mast cell reconstituted mast cell deficient mice show allergen induced airway hyper-responsiveness, whereas mast cell deficient mice are incapable of this response (Kobayashi *et al.,* 2000b). The increased cholinergic smooth muscle tone observed in response to incubation of equine small airways with mast cell mediators suggests a similar role for mast cells in inducing bronchospasm in equine heaves (Olszewski *et al.,* 1999).

Mast cell participation in the late phase response of allergic asthma is implicated by normal numbers of bronchial tissue mast cells in asthmatics at 4h, but increased numbers at 24h following allergen exposure, with the count at 24h significantly correlated with severity of response (Crimi *et al.,* 1991). Mast cell derived neutrophil and eosinophil chemotactic factors have also been associated with the development of the late phase inflammatory response (Nagy *et al.,* 1982). These findings are of interest given the apparent predominance of a late phase response in equine heaves, with intraluminal neutrophil accumulation not occurring until several hours following challenge.

1.4.4. Mast Cell Mediators

1.4.4.1. Tryptase

Tryptase, a neutral serine endopeptidase, is the predominant mast cell proteinase and exhibits trypsin-like specificity, preferentially cleaving peptide bonds at the carbonyl side of lysine or arginine residues, except when followed by a proline residue. Tryptase is considered to be mast cell specific as, although basophils contain small amounts of this proteinase, it is only approximately 0.2-0.4% of that present in mast cells (Castells *et al.,* 1987; Schwartz *et al.,* 1987).

The enzymatically active form of tryptase is a tetramer of 120-140kDa, consisting of four tryptase monomer subunits of 31-34kDa in a ring-like structure with a central pore (Pereira *et al.,* 1998). The protected location of the active sites facing inwards towards the central pore makes tryptase resistant to endogenous mammalian

proteinase inhibitors. The tryptase tetramer is stabilised by heparin and other negatively charged proteoglycans with the monomers becoming inactive upon dissociation. The tetrameric form of tryptase is also stabilised *in vitro* by hydrophobic interactions at high salt concentrations (>0.5M). The stability of the tetramer is thought to govern tryptase activity *in vivo* such that if heparin is scavenged by substances with a higher affinity, for example neutrophil lactoferrin, the tetramer dissociates into inactive monomers (Schwartz and Bradford, 1986; Addington and Johnson, 1996). Therefore, interestingly, neutrophil recruitment in heaves affected horses may actually have a role in restricting tryptase activity in the airway.

Multiple tryptase subtypes have been recognised in some species such as humans and sheep and different functional roles have been elucidated for human α- (constitutive) and β- (inducible) tryptases (Miller *et al.,* 1989; Miller *et al.,* 1990; Huang *et al.,* 2001). An equine tryptase, which shows strong identity with human tryptase-β over the first 20 residues of the N-terminal amino acid (Aa) sequence, has recently been isolated from equine mastocytoma tissue (Pemberton *et al.,* 2001).

As previously indicated, the majority of pulmonary mast cells are MC_T making tryptase abundantly available in the lung. A role for tryptase in human asthma has been suggested by elevated sputum (Pizzichini *et al.,* 1996) and BALF tryptase concentrations in asthmatics compared to control subjects (Wenzel *et al.,* 1988; Broide *et al.,* 1991; Bousquet *et al.,* 1991). Asymptomatic asthmatics also have higher BALF tryptase concentrations than control subjects, implying some degree of ongoing mast cell activation in the asymptomatic asthmatic airway (Broide *et al.,* 1991; Jarjour *et al.,* 1991). These tryptase concentrations in airway biological fluids are positively correlated with level of challenge and severity of clinical symptoms (Alvarez *et al.,* 2000). Although some authors have reported increased numbers of mast cells in the BALF compartment of asthmatic subjects (Tomioka *et al.,* 1984; Walls *et al.,* 1990), most studies report elevated BALF tryptase concentrations without a concurrent increase in the intraluminal mast cell count (Wardlaw *et al.,* 1986; Wenzel *et al.,* 1988; Chan-Yeung *et al.,* 1989; Broide *et al.,* 1991). This

suggests that the increased numbers of degranulating mast cells observed in the bronchiolar epithelium of human asthmatics may contribute to BALF proteinase concentrations.

Tryptase has potent biological activities, which are believed to contribute to the inflammatory response and hyper-reactivity of the asthmatic airway. Incubation of guinea pig skin with tryptase causes a dose-dependent increase in microvascular permeability which is similar in magnitude, but has a longer duration of action, than that induced by histamine (He and Walls, 1997). Despite this difference in time-course, it seems likely that induction of microvascular leakage is mediated, at least in part, by histamine because pre-treatment with histamine receptor antagonists markedly inhibits both tryptase and histamine induced effects on guinea pig skin (He and Walls, 1997).

Another potential role for tryptase in the asthmatic airway is in the development of bronchospasm. Incubation of human bronchial tissue with tryptase *in vitro* causes migration of mast cells from the epithelium into the airway submucosa and smooth muscle (Berger *et al.,* 1999). This is thought to be biologically significant as tryptase has a potent direct and indirect agonist action on human airway smooth muscle cells (Chambers *et al.,* 2001; Berger *et al.,* 2001b). The action of tryptase on equine airway smooth muscle warrants investigation since other mast cell mediators (histamine, serotonin and LTD_4) have been shown to augment equine small airway cholinergic tone (Olszewski *et al.,* 1999). Tryptase has also been implicated in chronic airway remodelling by inducing the proliferation of human airway smooth muscle cells (Berger *et al.,* 1999; Berger *et al.,* 2001a; Brown *et al.,* 2002; Cho *et al.,* 2003) and synthesis of type I collagen in human lung parenchyma and airway fibroblasts *in vitro* at concentrations likely to be achieved *in vivo* (Cairns and Walls, 1997; Akers *et al.,* 2000). These actions are thought to be mediated via the G-protein coupled receptor proteinase activating receptor-2 (PAR-2), as similar magnitude responses can be elicited using PAR-2 agonists (Akers *et al.,* 2000; Chambers *et al.,* 2001; Berger *et al.,* 2001a). In addition, immunohistochemical staining has localised PAR-2 to the surface of human lung fibroblasts (Akers *et al.,* 2000).

Tryptase is also hypothesised to play a functional role in regulation of neurogenic airway inflammation. Tryptase degrades the endogenous bronchodilator vasoactive intestinal peptide (VIP) and is suggested to be responsible for the decreased levels of VIP observed in the airway nerves of asthmatics and their consequent increased bronchial responsiveness (Tam and Caughey, 1990). The rapid rate of hydrolysis of calcitonin gene related peptide by tryptase suggests that it may terminate the potent effects of this neuropeptide on bronchial and vascular smooth muscle tone and permeability (Tam and Caughey, 1990). Activation of pre-kallikrein and generation of kinins (endogenous peptides causing increased vascular permeability, smooth muscle contraction and neutrophil chemotaxis), is also mediated by tryptase (Pasquale *et al.,* 1991; Imamura *et al.,* 1996).

Actions of tryptase in the epithelial microenvironment suggest a pivotal role in neutrophil recruitment. Biologically relevant concentrations of tryptase stimulate bronchiolar epithelial cells to upregulate expression of ICAM-1 and to increase secretion of IL-8 (Cairns and Walls, 1996). Furthermore, tryptase itself is also reported to be chemotactic for human peripheral blood neutrophils (Walls *et al.,* 1995). Injection of tryptase into guinea pig skin and mouse peritoneum induced marked neutrophil accumulation within 6h and this recruitment was prevented by co-injection of tryptase inhibitors (He *et al.,* 1997). This neutrophil recruitment over a time course pertinent to that observed following challenge of heaves susceptible horses invites speculation of a similar role for tryptase in equine heaves.

In a self-amplification role, tryptase induces further mast cell activation causing concentration dependant histamine release from human tonsillar and lung mast cells (He and Walls, 1997; He *et al.,* 1998). Furthermore, tryptase has been shown to potentiate histamine induced contraction of sensitised human (Johnson *et al.,* 1997; Berger *et al.,* 1999), guinea pig (Barrios *et al.,* 1998) and canine (Sekizawa *et al.,* 1989) bronchial smooth muscle *in vitro* and of allergic sheep *in vivo* (Molinari *et al.,* 1996). These studies provide strong evidence for a link between tryptase, histamine

and airway hyper-responsiveness across different species such that a similar effect may be present in equine heaves.

A number of studies have also investigated modification of the allergic airway response with tryptase inhibitors. In a mouse model of asthma, Oh *et al.* (2002) found that MOL 6131 (a potent specific tryptase inhibitor) reduced airway luminal total cell count and eosinophilia, airway tissue eosinophilia, goblet cell hyperplasia, mucus secretion and peribronchiolar oedema and also inhibited the release of IL-4 and IL-13 into BALF, although it did not alter airway hyper-reactivity. The tryptase inhibitors, aprotinin, APC-366 [N-(1-hydroxy-2-napthoyl)-L-arginyl-L-prolinamide hydrochloride], BABIM [bis(5-amidino-2-benzimidazolyl)methane], AMG-126737 [1,5-bis-{4-[(3-carbamimidoylbenzenesulfonylamino)-methyl]-phenoxy}-pentane] and endogenous secretory leukocyte proteinase inhibitor (SLPI) and lactoferrin decreased inducible bronchoconstriction and hyper-responsiveness in a model of allergic asthma in sheep *in vivo* (Clark *et al.,* 1995; Tanaka *et al.,* 1995; Molinari *et al.,* 1996; Elrod *et al.,* 1997; Barrios *et al.,* 1998; Wright *et al.,* 1999a; Wright *et al.,* 1999b). SLPI and APC 366 also inhibited tryptase mediated hyper-responsiveness of isolated guinea pig bronchi and attenuated the hyper-responsiveness in airway smooth muscle from antigen-sensitised animals subjected to antigen exposure *in vitro* (Barrios *et al.,* 1998). The inhibition of late phase airway responses by SLPI, lactoferrin, APC 366 and AMG-126737 in allergic sheep *in vivo* (Clark *et al.,* 1995; Elrod *et al.,* 1997; Wright *et al.,* 1999a; Wright *et al.,* 1999b; Krishna *et al.,* 2001) is particularly exciting given the suggested late phase response in equine heaves indicated by elevated PELF histamine concentrations 5h following challenge of heaves susceptible horses (McGorum *et al.,* 1993c). Interestingly, endogenous trypsin inhibitor activity of equine tracheal lavage fluid is reduced in horses with heaves, suggesting increased consumption or decreased production of inhibitors (Sandholm *et al.,* 1990; Maisi *et al.,* 1994). Although the specificity of these trypsin inhibitors may not include tryptase, it does suggest that proteolytic systems are involved in the pathogenesis of equine heaves.

1.4.4.2. Chymase

The other major mast cell proteinase is chymase, which has chymotrypsin-like activity preferentially cleaving C-terminal to aromatic and leucine residue sites. Some chymases however, such as sheep mast cell proteinase-1 (SMCP-1) and human cathespin G, possess dual specificity with both chymotryspin-like and trypsin-like activity (Pemberton *et al.,* 1997; Polanowska *et al.,* 1998). A β-chymase has recently been purified from equine mastocytoma tissue and termed equine mast cell proteinase-1 (eq.MCP-1) (Pemberton *et al.,* 2001). This proteinase shares 95% identity over the first 20 residues of the N-terminal Aa sequence with the cytotoxic T lymphocyte derived chymase human granzyme H (Pemberton *et al.,* 2001).

Mammalian mast cells may express multiple closely-related chymases which are members of a family of proteinases with varying substrate specificities expressed by granulocytes (Miller and Pemberton, 2002; Zamolodchikova *et al.,* 2003). This review of the potential biological effects of chymase is focused upon human α-chymase and therefore in the following section, 'chymase' refers to human α-chymase, unless otherwise stated.

Within the airway, the distribution of chymase containing mast cells is greatest near submucosal mucus glands (Matin *et al.,* 1992). This appears to be a pertinent location as chymase markedly stimulates mucus secretion, in a concentration dependent manner, from cultured bovine airway submucosal gland serous cells (Sommerhoff *et al.*, 1989a). Indeed, chymase is the most potent secretagogue for cultured serous cells, with respect to threshold concentration and magnitude of response identified to date, and this response can be blocked by chymase inhibitors (Sommerhoff *et al.*, 1989a). Furthermore, asthmatics have increased numbers of mast cells in close proximity to mucus glands compared to control subjects and mast cell number is also significantly correlated with the extent of airway lumen mucus plugging (Carroll *et al.,* 2002b). This potent secretagogue activity of chymase is therefore thought to play an important role in the exaggerated mucus secretion in asthma (Sommerhoff *et al.*, 1989a) and by analogy, may be involved in mucus hypersecretion in equine heaves.

Chymase, like tryptase, has potent biological effects, which may be of significance in the neuroregulation of airway secretion, vascular permeability and airway bronchomotor tone. Canine mast cell chymase is capable of inactivating peptides such as bradykinin (Reilly *et al.,* 1985), VIP and substance P (Caughey *et al.,* 1988). Similarly, human lung and skin mast cell chymase converts angiotensin I to angiotensin II with the same efficiency as angiotensin converting enzyme and this appears to be a biologically significant alternative pathway of activation (Reilly *et al.,* 1982; Wintroub *et al.,* 1984; Wintroub *et al.,* 1986). Angiotensin II contracts smooth muscle and enhances vascular permeability *in vitro* and may act to regulate these processes in the airway tissue microenvironment. In contrast however, eq.MCP-1 can hydrolyse substance P but can only very slowly cleave angiotensinogen and has no effect on angiotensin I and bradykinin, such that its substrate specificity appears to be highly restricted (Pemberton *et al.,* 2001).

Although chymase does not directly induce microvascular leakage, it greatly augments histamine induced cutaneous wheal formation in allergic dogs in a dose dependent manner (Rubinstein *et al.,* 1990). In contrast to the direct agonist action of tryptase, chymase is postulated to potentiate wheal formation by degradation of extracellular matrix components, thereby allowing freer flow of extravasated fluid (Vartio *et al.,* 1981; Briggaman *et al.,* 1984; Rubinstein *et al.,* 1990). However, this degradation could also be explained by an indirect effect of chymase via activation of matrix metalloproteinases (MMP) (Fang *et al.,* 1997).

Chymase appears to have a complex role in the regulation of mast cell degranulation, as although pre-incubation of mast cells with purified chymase did not induce histamine release, pre-incubation with specific chymase inhibitors decreased IgE dependent histamine release by as much as 80% (He *et al.,* 1999). This suggests a role for endogenous chymase in promoting IgE dependent degranulation. In an interesting contrast to the self amplification role of tryptase, chymase may also provide negative feedback to restrict further mast cell degranulation, as incubation of

human mast cells with purified chymase suppressed subsequent IgE dependent activation (He *et al.,* 1999).

1.4.4.3. Histamine

The biologic amine histamine is present in all mast cells but is not mast cell specific, also being present in equal quantity in basophils. Histamine exerts potent pro-inflammatory actions such as capillary dilation, increased capillary permeability and smooth muscle contraction which are generally mediated via type 1 histamine (H_1) receptors (White, 1990). Additional immunoregulatory effects such as inhibition of lymphocyte proliferation, neutrophil chemotaxis and increased mucus secretion are mediated via histamine type 2 (H_2) receptors (White, 1990; Tamaoki *et al.,* 1997). More recent evidence suggests a further role for histamine in cytokine regulation via type 2 and 3 (H_3) receptors (Jeannin *et al.,* 1994; Delneste *et al.,* 1994; Bissonnette, 1996; Krouwels *et al.,* 1998; Sirois *et al.,* 2000).

Elevated BALF histamine concentrations are present in asymptomatic and symptomatic asthmatics compared to control subjects (Casale *et al.,* 1987a; Wenzel *et al.,* 1988; Casolaro *et al.,* 1989; Chan-Yeung *et al.,* 1989; Broide *et al.,* 1991; Jarjour *et al.,* 1991). Furthermore, these BALF histamine levels are correlated with mast cell count, severity of disease and mechanical measurements of airway obstruction (Wardlaw *et al.,* 1988; Walls *et al.,* 1990; Jarjour *et al.,* 1991). Similarly, increased PELF histamine concentrations have been demonstrated in heaves susceptible horses following challenge (McGorum *et al.,* 1993c). Furthermore, the airways of both asthmatic (Casale *et al.,* 1987a; Durham *et al.,* 1988; Jarjour *et al.,* 1991) and heaves affected horses (Derksen *et al.*, 1988) are hyper-responsive to histamine, thereby increasing the biological relevance of any histamine release. The use of H_1 antagonists in asthmatics results in 30-50% attenuation in airway response to allergen (Finnerty *et al.,* 1989; Phillips and Holgate, 1989) suggesting that, although histamine may contribute to pathogenesis of disease, it is not the sole mediator of the asthmatic response.

Histamine stimulates the release of neuropeptides, such as Substance P, which may contribute to further mast cell activation via non-immunological means (Joos *et al.,* 2000). Interestingly, histamine also appears to be involved in cytokine regulation increasing synthesis and release of IL-10 (Sirois *et al.,* 2000), IL-5, IL-6 and IL-8 (Jeannin *et al.,* 1994) and inhibiting the release of IL-1 (Dohlsten *et al.,* 1988), IL-2, IFN-γ (Krouwels *et al.,* 1998), and TNF-α (Bissonnette, 1996). Histamine also stimulates CD8+ T cells and airway epithelial cells to produce IL-16, an early chemotactic factor for CD4+ T lymphocytes, (Mashikian *et al.,* 1998; Sirois *et al.,* 2000).

1.4.4.4. Cytokines

As can be seen from fig. 1.1, mast cells have the potential to produce a wide array of cytokines. Unlike most other cells, the mast cell contains preformed cytokines in granules ready for release upon mast cell activation. It appears that there is heterogeneity of mast cells with regard to their cytokine, as well as proteinase, content (Bradding *et al.,* 1995). Furthermore, cytokine release may be restricted to specific time periods (early, late or chronic phase) following stimulation (Kobayashi *et al.,* 2000a). Mast cell cytokines are predominantly type 2 cytokines, which are critical to the development and maintenance of the asthmatic IgE driven response (Hart, 2001) and in particular, are thought to be involved in the pathogenesis of the late phase cellular allergic response (Kobayashi *et al.,* 2000a). Mast cell cytokine biology is extremely complex and is still incompletely defined and therefore, only a brief overview of their putative involvement in airway inflammation is detailed below.

Several mast cell cytokines have been demonstrated to play an pivotal role in neutrophil recruitment, including IL-4, TNF-α, ENA and IL-8 (Boey *et al.,* 1989; Moller *et al.,* 1993; Strieter *et al.,* 1993; Teran *et al.,* 1995; Echtenacher *et al.,* 1996; Malaviya *et al.,* 1996; Girard *et al.,* 1997). As well as stimulating neutrophil recruitment, IL-4 also enhances neutrophil phagocytosis and increases neutrophil longevity by delaying apoptosis (Girard *et al.,* 1997). The importance of mast cell derived cytokines in the asthmatic response is demonstrated by airway mucosa from

asthmatics having greatly increased numbers of IL-4 and TNF-α positive mast cells compared to control subjects (Bradding *et al.,* 1994; Bradding, 1996). Furthermore, elevated BALF TNF-α (Broide *et al.,* 1992) and IL-8 (Teran *et al.,* 1995) concentrations have been reported in asthmatics. Interestingly, elevated BALF IL-8 concentrations have also been detected in heaves affected horses, with chemotactic activity correlated to the level of dust exposure (Franchini *et al.,* 1998). IL-8 is not expressed constitutively by mast cells and maximal mRNA expression of IL-8 takes up to 4h following mast cell activation (Moller *et al.,* 1993). This lack of pre-formed IL-8 may, in part, explain the time lag between challenge and cellular response in neutrophil mediated respiratory diseases such as occupational asthma and heaves. IL-8 can resist mild proteolytic degradation and therefore once released, is likely to have prolonged biological action (Strieter *et al.,* 1993). Mast cells are the only known cell to store preformed TNF-α in cytoplasmic granules and in models of acute septic peritonitis and pneumonia, mast cell derived TNF-α was critical in the rapid recruitment of neutrophils (Echtenacher *et al.,* 1996; Malaviya *et al.,* 1996). The release of preformed mast cell TNF-α has also been demonstrated to act as positive feedback to augment NF-κB transcription and production of further mast cell cytokines, including IL-8 (Coward *et al.,* 2002). TNF-α also stimulates bronchial epithelial cells and alveolar macrophages to upregulate IL-8 expression (Cairns and Walls, 1996). Lastly, TNF-α facilitates neutrophil recruitment by upregulating expression of endothelial cell derived neutrophil adhesion molecules including leukocyte adhesion molecule 1 (E-selectin) and ICAM-1 (Strieter *et al.,* 1993).

There are several other proposed roles for mast cell cytokines in the airway. Mast cell IL-4 and IL-5 mRNA and protein expression have been demonstrated in asthmatic bronchial biopsies (Ying *et al.,* 1997) where IL-5 is considered to have a primary role in late phase airway eosinophilic influx in asthma (Jaffe *et al.,* 1995). TNF-α can activate MMP-9, which may be involved in the fibrotic response of chronic asthma (Han *et al.,* 2002), and can also induce bronchial hyper-responsiveness (Wheeler *et al.,* 1990). Mast cell derived IL-4 induces IgE production from B cells independently of T cells, whereas maintenance of IgE synthesis in the asthmatic airway is believed to be dependent on IL-13 derived both from mast cells

and T lymphocytes (Pawankar *et al.,* 1997). IL-13 is also believed to stimulate mucus hypersecretion from goblet cells (Hart, 2001). Mast cell release of IL-4 and IL-16, augmented by histamine stimulation of IL-16 secretion from other cells as previously mentioned, also contribute towards T helper cell accumulation (Mashikian *et al.,* 1998).

1.5. Aims of This Project

Key roles for the mast cell, and in particular the mast cell proteinase tryptase, have been elucidated in the pathogenesis of both human allergic asthma and occupational asthma. Due to the similarities between these diseases and equine heaves, analogous mast cell involvement in the pathogenesis of equine heaves is proposed. This project was therefore designed to determine whether mast cells, and in particular mast cell proteinases, play a role in heaves by:

- Cloning and sequencing the equine mast cell serine proteinases equine tryptase and eq.MCP-1 in order to rationalise their enzymic activity and to develop molecular tools to probe mast cell proteinase expression in tissues from control and heaves affected horses
- Quantifying levels of mast cell serine proteinases in BALF from control horses and heaves susceptible horses (during both disease exacerbation and remission)
- Investigating the distribution and proteinase heterogeneity of equine mast cells in pulmonary tissues from control and heaves affected horses
- Probing mast cell proteinase mRNA transcript regulation in airway luminal and tissue mast cells from control and heaves susceptible horses in response to inhalational challenge *in vivo*
- Determining if a suspension of hay dust can induce release of mast cell proteinases from airway luminal cells from control and heaves susceptible horses *in vitro*
- Determining the effects of mast cell proteinases on cultured equine tracheal explants *in vitro*

Chapter 2: Purification and Validation of Antibodies

2.1. Summary

Previously produced polyclonal rabbit anti-equine tryptase and rabbit anti-eq.MCP-1 were cross-absorbed against albumin and affinity purified. These antibodies recognised equine tryptase and eq.MCP-1 respectively when used to probe western blots of mastocytoma extract or purified proteinases. Immunohistochemical staining of tissues with these antibodies successfully demonstrated tryptase and eq.MCP-1 positive mast cells. Furthermore, dual immunofluorescent labelling of mast cells in lung and colon tissue with these antibodies confirmed that these antibodies did not co-localise in dual positive cells and therefore detected mast cell proteinases in different granules.

2.2. Introduction

Mast cells contain many potent mediators including serine proteinases, biological amines, prostaglandins, leukotrienes and cytokines. Serine proteinases are the major protein constituents of the mast cell and are hypothesised to have a significant biological effect in the lung and other tissues (Caughey, 1991). Indeed, there is now considerable evidence confirming a role for the mast cell in the pathogenesis of human asthma, including occupational dust induced asthma (Casale *et al.,* 1987b; Chan-Yeung *et al.,* 1987; Wenzel *et al.,* 1988; Crimi *et al.,* 1991; Park *et al.,* 1998b; Park *et al.,* 1999; Holgate, 2000; Hart, 2001; Carroll *et al.,* 2002a). Equine heaves is similar in many respects to occupational dust induced asthma, since both are characterised by reversible neutrophilic pulmonary inflammation in response to inhaled organic dust (Enarson *et al.,* 1985; Huy *et al.,* 1991; Chan-Yeung *et al.,* 1992; Pirie *et al.,* 2002a). Consequently, speculation has arisen that mast cells may also play a role in the pathogenesis of equine heaves (McGorum *et al.,* 1993c; Hare *et al.,* 1999).

The serine proteinases equine tryptase (trypsin-like) and eq.MCP-1 (chymotrypsin-like) have previously been isolated from equine mastocytoma tissue and antibodies to these proteinases raised in rabbits (Pemberton *et al.,* 2001). Purification and validation of these antibodies was performed to allow further investigation of the role of the mast cell and their serine proteinases in equine heaves. Specifically, these antibodies would allow development of ELISAs for quantification of proteinases in equine BALF from control and heaves affected horses. Furthermore, these antibodies would facilitate immunohistochemical detection and enumeration of mast cells in lung tissue, to assess whether there is mast cell recruitment to the lung during clinical disease.

2.3. Materials and Methods

2.3.1. Extraction of Mast Cell Proteinases from High Salt Extract of Mastocytoma Tissue

2.3.1.1. Tryptase Extraction

A high salt extract (HSE) (2M NaCl, 50mM MES pH 6.1, 0.1% Brij 35) of equine mastocytoma tissue containing mast cell proteinases had previously been prepared and stored at -70°C (Pemberton *et al.,* 2001). A 2ml aliquot of HSE was added to 6ml 50mM MES, pH 6.1, 0.1% Brij 35 (low salt buffer) to give a final salt concentration of 0.5M and filtered through a 5μm membrane (Millex SV, Sigma). A Hi Trap heparin affinity column (1ml, Pharmacia) was pre-equilibrated with low salt buffer and then loaded with diluted HSE using a high performance liquid chromatography system (Waters, Millipore). Tryptase in the mastocytoma extract bound to the heparin column whilst the mobile phase was retained for later eq.MCP-1 extraction. Heparin-bound tryptase was then eluted using a 0-2M salt gradient in 50mM MES, pH 6.1, 0.1% Brij 35. Tryptase activity of the eluted fractions and chymase activity of the flow-through were measured chromogenically as described in section 2.3.1.4. Samples showing high tryptase activity were concentrated using a Centricon YM10 filter device (Millipore) at 6000g for 70min at 4°C.

2.3.1.2. Eq.MCP-1 Extraction

Eq.MCP-1 had previously been extracted from the heparin column flow-through generated during tryptase purification from equine mastocytoma HSE and was available for use (Pemberton *et al.,* 2001). This purified enzyme had given a double band at 32.1kDa on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and hydrolysed the thioester chymotrypsin substrate succinyl-Phe-Leu-Phe thiobenzyl ester (FLF-SBzl), indicating chymase activity.

2.3.1.3. SDS-PAGE of Eluted Fractions

Samples of eluted fractions (5µl) were heated to 95°C with 15µl of reducing buffer for 5min and run on a 12% SDS separating gel, 0.375M Tris, pH 8.8, at 200V, for 40min with Tris / glycine pH 8.3 running buffer. The gel was stained with 0.25% Coomassie Brilliant Blue R250 (Sigma) in 25% methanol, 5% acetic acid, 70% water for 15min and then destained with 1 part acetic acid : 5 parts methanol : 1 part deionised water until bands could be visualised.

2.3.1.4. Measurement of Proteinase Activity

Proteinase activity of eluted fractions was measured spectrophotometrically (Beckmann DU650).

Tryptase fractions were diluted 1:10 in 0.1M Tris-HCl pH 8.0, 0.1M NaCl, 1.0M glycerol and 10µl of diluted sample added to 180µl of the same buffer containing 10µg/ml heparin. The spectrophotometer was blanked on this solution and then 10µl chromogenic substrate 2.5mM pyroGlu-Pro-Arg-*p*-nitroanilide (S2366, Quadratech, final concentration 0.125mM) in water added. The rate of activity was measured at 405nm.

Eq.MCP-1 activity was determined using 10µl of a 1:10 dilution of purified proteinase in 0.1M HEPES pH 7.5. This diluted sample was added to 170µl 0.1M HEPES pH 7.5, 10µl 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 10mM in DMSO) and 10µl FLF-SBzl (10mM in DMSO).

2.3.1.5. Preparation of Tryptase Column

Tryptase isolated from mastocytoma HSE was used to prepare a tryptase column for affinity purification of polyclonal anti-equine tryptase. Isopropanol was washed from a 1ml Hi Trap NHS activated column (Pharmacia) using 1mM ice cold HCl at a rate of approximately 1ml/min. Tryptase was loaded onto the column in binding buffer (0.2M $NaHCO_3$, 0.5M NaCl, pH 8.3) at the same rate and the column then sealed and left for 30min at room temperature. The column was then washed three times with 2ml 0.5M ethanolamine, 0.5M NaCl, pH 8.3 (buffer A), three times with 2ml 0.1M acetate, 0.5M NaCl, pH 4.0 (buffer B) and a further three times with buffer A. The column was left for 30min at room temperature and then washed three times each with buffer B, buffer A and again with buffer B. Finally the column was equilibrated with 2ml PBS.

2.3.2. Purification of Antibodies

2.3.2.1. Cross-Absorption of Antisera Against Albumin

Polyclonal antisera directed against equine tryptase or eq.MCP-1 had previously been produced at R(D)SVS and stored at -20°C (Pemberton *et al.,* 2001). An albumin column (Hi Trap NHS activated 1ml column, Pharmacia), to which 10mg equine albumin had previously been conjugated, was connected to the fast performance liquid chromatography unit (FPLC; Pharmacia LCC 500) and washed with 0.1M citric acid to release any protein pre-bound to the column. Rabbit anti-equine tryptase or anti-eq.MCP-1 antisera was thawed, diluted 1:1 with phosphate buffered saline (PBS) and filtered through a 0.45μm cellulose acetate membrane (Millipore). The diluted antisera was then loaded into the FPLC superloop and loaded onto the albumin column in 1.5ml aliquots. Contaminating anti-albumin antibodies bound to the albumin column, whilst anti-equine tryptase / anti-eq.MCP-1 antisera were collected in the flow-through. Bound albumin antibodies were eluted with 0.1M citric acid between loading of separate aliquots to prevent saturation of the column.

2.3.2.2. Affinity Purification of Antibodies

Albumin cross-absorbed anti-equine tryptase / anti-eq.MCP-1 serum was re-loaded into the FPLC superloop and injected onto an equine tryptase / eq.MCP-1 column. An eq.MCP-1 column was available from preliminary work leading up to this project (Pemberton *et al.,* 2001). Flow-through was discarded and affinity purified antibodies eluted from the column with 0.1M citric acid. Eluted antibodies were immediately buffered to approximately pH 7.0 with 1M Tris. The albumin-cross absorbed, affinity purified antibodies were then either concentrated and stored, or biotinylated.

2.3.2.3. Protein Concentration of Antibodies

Albumin-cross absorbed, affinity purified antibodies were loaded into a centrifugal filter device (Centricon YM10, Millpore) with a retention size of >10kDa and centrifuged at 4500g, 4°C, 30min. Absorbance of the concentrated antibodies was measured spectrophotometrically (Beckmann DU650) at 260 and 280nm and the protein concentrations calculated using the equation:

$$\text{Protein concentration (ng/ml)} = (1.55 \times A_{280}) - (0.76 \times A_{260})$$

Antibodies were then diluted 1:1 w/v with glycerol and stored at -20°C.

2.3.2.4. Biotinylation of Antibodies

Buffer exchange of antibodies was performed using a Hi Trap desalting column (Pharmacia) equilibrated with 0.1M $NaHCO_3$, pH 8.5. Albumin-cross absorbed, affinity purified antibodies in Tris buffer were loaded onto the column by syringe and then eluted using 0.1M $NaHCO_3$, pH 8.5. The protein concentration of eluted antibodies was measured using a spectrophotometer. Biotin (Long Arm) N hydroxysuccinimide ester (Vector Laboratories) (25mg/ml in DMSO) was added to the antibody in a glass bijoux such that the final ratio was 10% biotin:protein and the solution left at room temperature for 2h with occasional mixing by hand. Excess biotin was then removed by HPLC gel filtration using a Hi Trap 5ml desalting column (Pharmacia) with 5kDa cut off.

2.4. Validation of Antibodies

2.4.1. Western blotting of Mastocytoma HSE and Purified Proteinases

Equine mastocytoma tissue HSE, purified equine tryptase or purified eq.MCP-1 were diluted 1:10 with reducing buffer and heated to 95°C for 5min. Samples were run (0.1μg proteinase / 10μl diluted HSE per lane) on two 12% SDS separating gels, 0.375M Tris, pH 8.8, at 200V (Mini-Protean-II, Bio-Rad), for 40min with Tris / glycine pH 8.3 running buffer. One gel was stained with Coomassie Brilliant Blue R250 as previously described in section 2.3.1.3. The other gel was blotted (Trans-Blot SD, Bio-Rad) onto polyvinylidenfluoride (PVDF) membrane (Immobilon P, Millipore), 80mA for 1h, blocked for 1h with PBS / 0.5M NaCl, 0.5% Tween 80, 1% skimmed milk powder and then probed with 1μg/ml rabbit anti-equine tryptase / rabbit anti-eq.MCP-1 for 1h. The secondary antibody mouse monoclonal anti-rabbit IgG–alkaline phosphatase conjugate (clone RG-96, Sigma) was applied at 1/20,000 dilution for 1h, and then the reaction developed with substrate 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium (BCIP / NBT) (Sigma).

2.4.2. Immunohistochemistry

2.4.2.1. Equine Tissue Sections

Sections of equine duodenum and ileum previously collected from horses euthanased at R(D)SVS and fixed in 4% paraformaldehyde were available for immunohistochemical testing of antibodies.

2.4.2.2. Antigen Retrieval Techniques

As very poor staining was observed following immunohistochemical labelling of paraformaldehyde fixed tissue, several antigen retrieval methods were assessed.

a) Citrate Boiling

Following de-waxing in xylene and rehydration through graded alcohols, slides were immersed in boiling 10mM citrate, pH 6.0 under pressure for 4min (Biomen pressure

cooker, A. Menarini Diagnostics) in a microwave (Panasonic 800W D). This technique was found to cause excessive tissue destruction and therefore a modified technique using 10min immersion in 10mM citrate, pH 6.0 heated to 95-100°C on a magnetic stirring plate (Bibby Hotplate Stirrer B212) was also performed. Although this resulted in positive labelling of cells, some tissue destruction was still evident and therefore reduced exposure times of 1min and 5min and immersion in 10mM citrate, pH 6.0 for 10min at room temperature were employed. Whilst immersion in cold citrate buffer did not result in any tissue destruction, there was also no positive staining evident. Sections treated with even 1min boiling citrate buffer showed excessive tissue destruction (data not shown).

b) Trypsin Digestion

A successful method of antigen retrieval was found using trypsin digestion. Following de-waxing in xylene and rehydration through graded alcohols, slides were immersed in 0.1, 0.5 or 1mg/ml trypsin (Sigma) in PBS for 30min at 37°C. The concentration deemed most successful was 0.1mg/ml as this achieved good antigen retrieval without non-specific staining (data not shown).

2.4.2.3. Final Immunohistochemistry Protocol

All steps were performed at room temperature unless otherwise stated. Slides were de-waxed in xylene, rehydrated through graded alcohol and washed with tap water. Tissue sections were then incubated in 0.1mg/ml trypsin in PBS for 30min at 37°C, washed twice in PBS and then immersed in 97% methanol / 3% hydrogen peroxide for 10min to block endogenous peroxidase activity. Following a further wash with tap water, slides were placed in Sequenza carriers (Shandon) and incubated with blocking buffer (PBS / 0.5M NaCl / 0.5% Tween 80 [Sigma]) for 30min. All subsequent dilutions were made using this blocking buffer. Primary antibodies, polyclonal rabbit anti-equine tryptase or anti-eq.MCP-1, were then applied at 1µg/ml for 1h. Normal rabbit IgG (1µg/ml) was applied to control sections. Following washing with PBS, slides were incubated with biotinylated goat anti-rabbit IgG (1:400 dilution, Vector Laboratories) for 30min. Slides were again washed with PBS and then avidin-horseradish peroxidase conjugate (ABC kit, Vector Laboratories)

applied for 30min. Colour was developed using 3,3'-diaminobenzidine (DAB kit, Vector Laboratories) and counterstaining with Mayer's haematoxylin (Sigma). Slides were washed in Scott's tap water, dehydrated and mounted using DPX (Sigma).

2.4.3. Dual Immunofluorescent Labelling of Equine Tissues

Samples of colon and lung tissue were collected immediately post mortem from a horse euthansed at R(D)SVS for orthopaedic disease. Tissues were immersed in Carnoys Fixative for 24h and then transferred to 70% alcohol until further processing. Carnoys fixative is reported to be the optimal fixative for preservation of mast cells (Nicholls, 1978; Irani *et al.,* 1986; Craig *et al.,* 1986; Shanahan *et al.,* 1987; Winder and von Fellenberg, 1990; Kube *et al.,* 1998; Kuther *et al.,* 1998) and its use in equine tissues is discussed further in Chapter 5. Fixed samples were submitted to the Division of Veterinary Pathology at R(D)SVS for paraffin embedding and cutting of 4µm thickness serial sections onto coated slides (Surgipath).

Slides were de-waxed, rehydrated through graded alcohols and treated with DAB for 5min to quench eosinophil peroxidase. Non-specific binding was blocked by incubation with PBS / 0.5M NaCl / 0.5% Tween 80 (Sigma) / 10% normal donkey serum (Sigma) for 30min. Rabbit anti-equine tryptase was applied as the primary antibody at 1µg/ml with overnight (approx. 8h) incubation at 4°C. Normal rabbit IgG (1µg/ml) was used for control sections. Following washing with PBS, slides were incubated with donkey anti-rabbit IgG-Alexafluor 488 (Invitrogen) at 4µg/ml for 30min. This incubation and all subsequent steps were performed in the dark. Slides were washed with PBS and then blocked with PBS / 0.5M NaCl / 0.5% Tween 80 (Sigma) / 10% normal rabbit serum (Sigma) for the rest of the day. Biotinylated rabbit anti-eq.MCP-1 (2µg/ml) was applied overnight (approx. 8h) at 4°C. Biotinylated normal rabbit IgG was used on control slides. Following washing with PBS, slides were treated with ABC kit for 30min. Slides were again washed, incubated with tyramide-Alexafluor 568 (1:200 dilution, Molecular Probes) for 10min and then washed again and mounted with Mowiol.

Fluorescent images were acquired using an MRC-600 confocal laser scanning microscope (CLSM: Bio-Rad Laboratories) mounted on an Axiovert 100 inverted microscope equipped with Plan-Apochromat objective lenses (Carl Zeiss). Cells labelled with anti-equine tryptase fluoresced green under 488nm excitation whereas anti-eq.MCP-1 labelled cells fluoresced red under 568nm excitation.

2.5. Results

2.5.1. Tryptase Extraction from Mastocytoma HSE

Fractions eluted from the heparin affinity column that contained high levels of trypsin-like activity with S2366 were combined. SDS-PAGE of this product showed a triple band complex at approximately 32-35kDa, characteristic of equine tryptase (Pemberton *et al.,* 2001) (fig. 2.1).

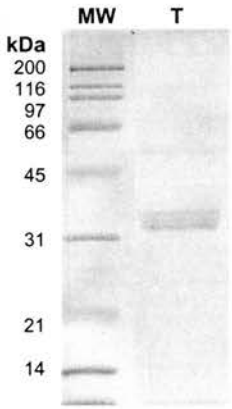

Fig. 2.1: Coomassie Brilliant Blue R250 stained SDS-gel showing the characteristic triple band of tryptase at approximately 32kDa. MW = molecular weight markers. T = 1μg equine tryptase. kDa = size of molecular weight markers in kilo Daltons.

2.5.2. Purification of Antibodies

The protein concentration of albumin cross-absorbed affinity purified polyclonal anti-eq.MCP-1 was 2.28mg/ml. The protein concentration of albumin cross-absorbed affinity purified polyclonal anti-equine tryptase was 1.80mg/ml. Biotinylated anti-eq.MCP-1 and biotinylated anti-equine tryptase had protein concentrations of 0.86mg/ml and 1.80mg/ml, respectively.

2.5.3. Validation of Antibodies

2.5.3.1. Western Blot Analysis

Affinity purified, albumin cross-absorbed, polyclonal rabbit anti-equine tryptase and anti-eq.MCP-1 positively identified purified equine tryptase and eq.MCP-1, respectively. The antibodies also detected these proteinases in equine mastocytoma HSE (fig 2.2). Control rabbit IgG was negative on Western Blot analysis.

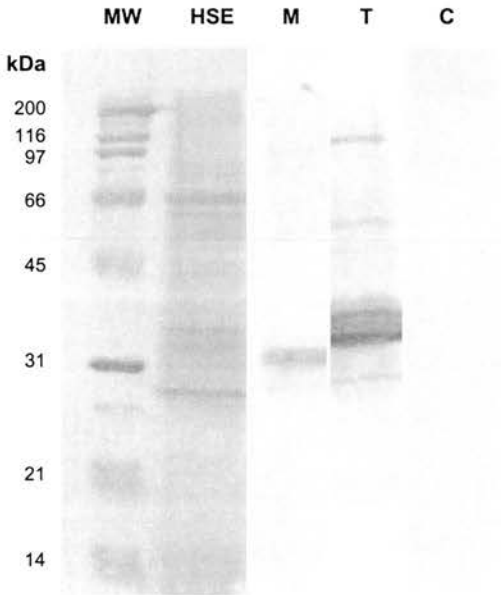

Fig. 2.2: Composite picture of the Western blot of high salt extract (HSE) of equine mastocytoma tissue. MW = Coomassie blue stained molecular weight markers. HSE = Coomassie blue stained HSE showing the characteristic triple band of tryptase at approximately 32kDa. M = HSE probed with anti-eq.MCP-1, T = HSE probed with anti-equine tryptase, C = HSE probed with normal rabbit IgG. kDa = size of molecular weight markers in kilo Daltons.

2.5.3.2. Immunohistochemistry

Affinity purified, albumin cross-absorbed, polyclonal rabbit anti-equine tryptase and anti-eq.MCP-1 successfully labelled mast cells in equine ileal sections following trypsin digestion (figs. 2.3, 2.4). Positively labelled cells showed deep brown colouration throughout the cell. For both anti-equine tryptase and anti-eq.MCP-1, most positively labelled cells were present in the ileal submucosa with only occasional positive mucosal cells identified.

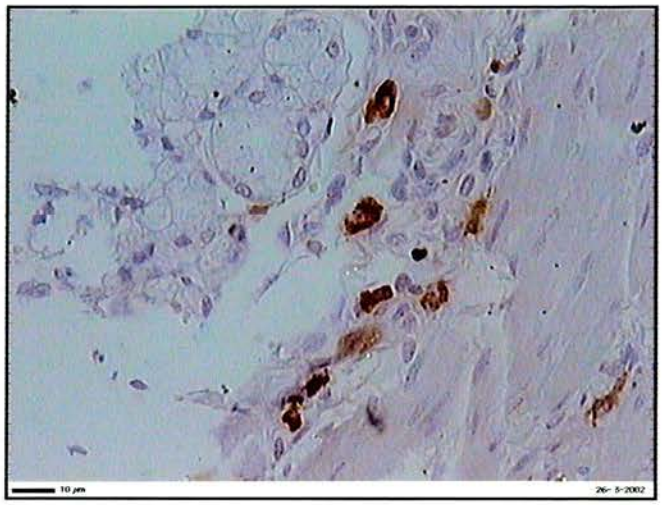

Fig. 2.3: Tryptase immunoreactive cells in paraformaldehyde fixed equine ileum. Size marker = 10μm.

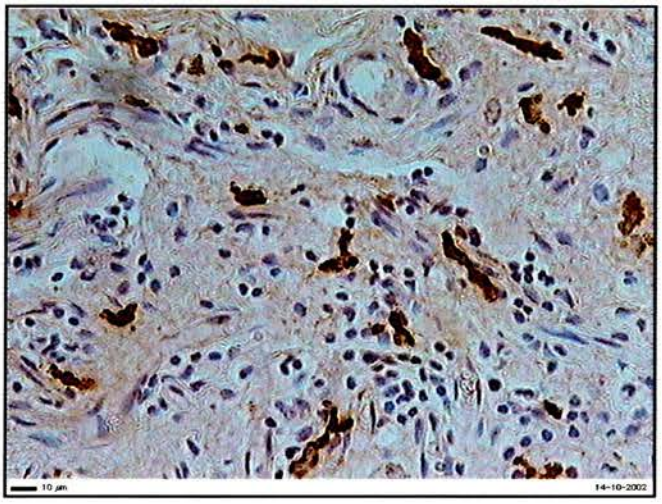

Fig. 2.4: Eq.MCP-1 immunoreactive cells in paraformaldehyde fixed equine ileum. Size marker = 10μm.

2.5.3.3. Dual Immunofluorescent Labelling

Colon sections showed many intensely labelled mast cells. Single and dual labelled cells were present with single eq.MCP-1 positive cells being predominant (fig. 2.5). In contrast, lung sections showed fewer positively labelled cells, with predominantly tryptase positive mast cells, and only occasional dual positive or solely eq.MCP-1 positive cells observed. All control sections were negative. It was clear on confocal microscopy that the two fluorescent labels did not co-localise.

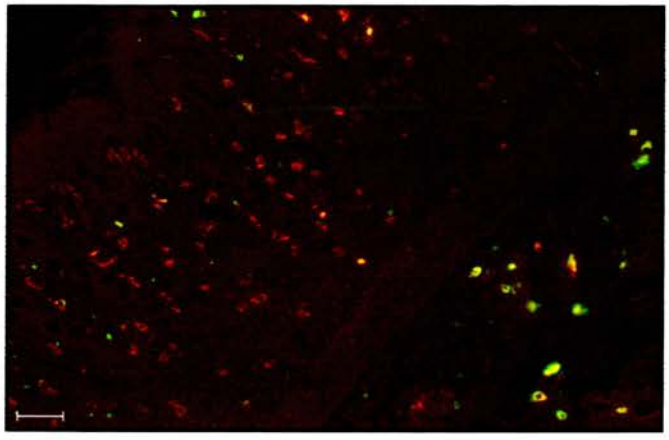

Fig. 2.5: Dual immunofluorescent labelling of equine colon. Cells fluorescing green are tryptase positive, cells fluorescing red are eq.MCP-1 positive, yellow cells are dual positive. Size marker = 10μm.

2.6. Discussion

2.6.1. Purification of Equine Tryptase

Tryptase purified from equine mastocytoma HSE hydrolysed the arginine containing chromogenic substrate S2366. This activity is characteristic of trypsin-like proteinases which cleave arginine and lysine residues unless followed by a proline residue (Schwartz, 1994). Active tryptase is stable as a tetrameric structure composed of four individual tryptase monomers of 31-34kDa (Schwartz, 1994). The purified proteinase showed a triple band at approximately 32-35kDa following SDS-gel electrophoresis representing multiple glycosylated forms of the tryptase subunit.

2.6.2. Purification and Validation of Antibodies

Cross-absorbed affinity purified anti-equine tryptase and anti-eq.MCP-1 successfully recognised purified proteinases and proteinases in equine mastocytoma tissue HSE. All bands visualised on SDS-gel electrophoresis were recognised on probing Western blots with these antibodies. Validation of the biotinylated antibodies was performed as part of the development process for the ELISAs, and as such is described in Chapter 4.

The anti-equine tryptase and anti-eq.MCP-1 antibodies successfully detected tryptase and eq.MCP-1 positive cells in equine tissues using immunohistochemical and

immunofluorescent techniques. Antigen retrieval was required for the immunohistochemical demonstration of mast cells in paraformaldehyde fixed tissues. Trypsin digestion was clearly superior to citrate boiling in improving epitope recognition without resulting in tissue destruction. Antigen retrieval techniques appear to be more successful in the submucosa than the mucosa, as positive mucosal cells, which are reported in other species (Huntley *et al.,* 1985), were rarely observed, even following trypsin digestion. This was considered likely to be an artefact due to fixation with paraformaldehyde because formalin based fixatives are reported to result in underestimation of mucosal mast cells across species (Nicholls, 1978; Irani *et al.,* 1986; Craig *et al.,* 1986; Shanahan *et al.,* 1987; Winder and von Fellenberg, 1990; Kube *et al.,* 1998; Kuther *et al.,* 1998). Although not ideal, paraformaldehyde fixed tissues were used in this instance as sections were readily available and eliminated the necessity to procure tissues. Furthermore, the use of antigen retrieval in paraformaldehyde fixed equine tissues had previously resulted in successful recognition of tryptase and eq.MCP-1 positive mast cells with these antibodies (Pemberton *et al.,* 2001). Abundant mucosal tissue mast cells have been identified by electron microscopy and other staining techniques in horses (Mair *et al.,* 1988) and other species (Guerzon *et al.,* 1979; Fox *et al.,* 1981; Huntley *et al.,* 1985; Jolly *et al.,* 2000), therefore their absence from paraformaldehyde fixed tissue was attributed to a fixative effect rather than a true absence of mast cells from this location. This observation led to the use of Carnoys fixative for tissues intended for dual immunofluorescent labelling. Plentiful mucosal mast cells were identified in these Carnoys fixed tissues, further supporting their absence in paraformaldehyde tissue being an artefact related to paraformaldehyde fixation.

Dual immunofluorescence was performed to confirm the cellular origin of eq.MCP-1. Whilst tryptase is considered to be a mast cell specific proteinase (Schwartz, 1994), chymotrypsin-like proteinases have been isolated from both mast cells and T lymphocytes (Peitsch and Tschopp, 1994). The mast cell origin of eq.MCP-1 was therefore confirmed by dual labelling of cells with both proteinases. The presence of single labelled cells and the lack of co-localisation of labels in dual positive cells demonstrated that the antibodies recognised proteinases in separate storage granules.

2.7. Conclusion

Albumin cross-absorbed, affinity purified anti-equine tryptase and anti-eq.MCP-1 were validated to successfully recognise their respective proteinases in equine mastocytoma extracts and in equine tissues. These antibodies were purified to allow further investigation into the role of the mast cell in the pathogenesis of equine heaves. Their use in the development of ELISAs allowed determination of BALF proteinase concentrations for control and heaves horses (Chapter 4). Furthermore, possible mast cell recruitment to the lung in heaves affected horses was investigated (Chapters 4 and 5) by immunohistochemistry and immunofluorescence of equine mast cells in lung tissue and BALF cytospin preparations.

Chapter 3: Cloning and Sequencing of Equine Mast Cell Proteinases and their Expression in Equine Tissues

3.1. Summary

It was attempted to clone and sequence the mast cell proteinases equine tryptase and eq.MCP-1 from equine tissues. The amino acid (Aa) sequence deduced from the cDNA nucleotide sequence for equine tryptase shared strong identity with other tryptases; 77% with human tryptase β1 and 73% with sheep tryptase-2. The deduced N-terminal Aa sequence corresponded exactly with that determined from tryptase purified from equine mastocytoma tissue and the peptide mass fingerprint of mastocytoma derived equine tryptase was consistent with the cloned equine tryptase sequence. Unusually for a trypsin-like proteinase, equine tryptase has alanine at residue 216, rather than glycine, which confers increased arginine substrate specificity and may restrict fibrinogenolysis *in vivo*. Cloned putative eq.MCP-1 has an uncharged residue (glutamine) at 226 conferring chymase (chymotrypsin-like) substrate specificity. The cloned Aa sequence shared greatest identity (65%) with the cytotoxic T lymphocyte associated serine esterase human granzyme B, 57% identity with the putative chymase sheep mast cell proteinase-3 and 56% identity with dual specific sheep mast cell proteinase-1. The N-terminal Aa sequence of eq.MCP-1 isolated from mastocytoma tissue differed from that determined from the cDNA nucleotide sequence by 2 residues. Furthermore, peptide mass fingerprinting of eq.MCP-1 isolated from equine mastocytoma tissue shared only 3 peptides with the cloned putative eq.MCP-1 sequence. Granulocytes of most mammalian species express multiple closely-related proteinases and therefore it is likely that a similar but novel chymase was sequenced. Expression of the cloned proteinases in equine tissues was assessed by reverse transcriptase polymerase chain reaction (RT-PCR). Both tryptase and putative eq.MCP-1 were expressed in bronchial tissue, bronchiolar tissue, liver, skin and colon.

3.2. Introduction

Two neutral serine proteinases have previously been extracted from equine mastocytoma tissue, namely equine tryptase, which has trypsin-like activity, and eq.MCP-1 which has chymotrypsin-like specificity. Eq.MCP-1 demonstrated extremely restricted substrate specificity, which most closely resembled that of the cytotoxic T lymphocyte serine proteinase human granzyme H (Pemberton *et al.*, 2001).

Tryptases and chymases possess a wide range of potent biological effects which contribute to inflammatory processes, particularly in allergic disease (Denburg *et al.*, 1989; Holgate, 2000). Equine tryptase and eq.MCP-1 positive mast cells have previously been identified in the equine lung (Pemberton *et al.*, 2001) and therefore these proteinases may have relevance in the equine airway with regard to the pathogenesis of heaves.

The aim of this study was to clone and sequence equine tryptase and eq.MCP-1 and to assess their expression in various equine tissues. Cloning and sequencing of these proteinases will allow further rationalisation of their enzymic activity and the development of molecular tools to probe transcription of mast cell proteinases in control and heaves-affected horses.

3.3. Materials and Methods

3.3.1. Samples for Cloning and Sequencing of Proteinases

Colonic tissue was collected into RNA-Later (Ambion) immediately post mortem from one horse euthanased for orthopaedic disease. Tissue was stored at 4°C overnight and then -20°C until further processing. Cell pellets were harvested (400g, 10min) from BALF samples collected from 2 horses referred to R(D)SVS clinical hospital for poor exercise performance. Cells were resuspended in 1ml TRI Reagent (Sigma) by vortexing vigorously and then incubated at room temperature for 5min before repeated vortexing. Cell pellets were stored at -70°C prior to RNA extraction.

3.3.2. RNA extraction

Approximately 100mg colon tissue was homogenised on ice with 2ml of TRI Reagent (Sigma). The homogeniser probe was soaked in Neutracon (Decon Laboratories Ltd.) overnight prior to use and cleaned thoroughly between samples. Molecular biology grade chloroform (0.2ml, Sigma) was added to homogenised tissue or thawed BALF cell pellets in 1.5ml eppendorfs. Samples were vortexed for 30s, allowed to stand at room temperature for 5min and then centrifuged at 13,000rpm, 15min (Biofuge, Kendro Laboratory Products). The upper aqueous phase was carefully collected and 650µl isopropanol added to precipitate total RNA. Following incubation at room temperature for 10min, RNA was pelleted by centrifugation at 13,000rpm, 15min. Isopropanol was removed and the RNA pellet washed with 1ml 75% ethanol by vortexing and then centrifugation at 8,000rpm, for 5min. The RNA pellet was air-dried following removal of ethanol and then dissolved in 100µl RNase free water (Ambion). RNA concentration and purity were measured by spectrophotometer (Beckmann DU650).

DNase treatment of TRI Reagent extracted RNA was performed using DNA-free DNase treatment kit (Ambion). RNA (5µg in a volume of 41µl) was incubated for 1-2h, at 37°C, with 4µl DNase I and 5µl DNase I buffer and then 5µl DNase I inactivation reagent was added and the reaction incubated at room temperature for 2min. The inactivation reagent was then pelleted by centrifugation for 1min, at 13,000rpm.

3.3.3. Reverse Transcription of RNA

DNA free total RNA was reverse transcribed using random hexamer primers and avian myeloblastosis virus (AMV) reverse transcriptase (Reverse Transcription System, Promega). The following reaction was set up on ice; 4µl 25mM $MgCl_2$, 2µl 10x reverse transcription buffer (100mM Tris HCl, pH 8.8), 2µl 10mM dNTPs, 0.5µl (20u) RNase inhibitor, 0.5µl (15u) AMV-RT, 1µl (0.5µg) random hexamers and 10µl RNA. The reaction mixture was incubated for 10min at room temperature, 50 min at 42°C and finally 5min at 99°C to stop the reaction (Technegene, Techne). The

mixture was cooled on ice, diluted to 100µl with RNase free water and the cDNA concentration measured by spectrophotometry.

3.3.4. Design of Primers

Sheep tryptase primers (kindly donated by S. McAleese, R[D]SVS) in a highly conserved region of the proteinase were used for initial isolation of an equine tryptase sequence fragment. Eq.MCP-1 primers were designed from well conserved areas of other known chymases using alignment analysis (Clustal W, EMBL EBI). Following sequencing of these initial fragments, specific primers for the 3' and 5' regions of each proteinase could be designed using the software programme Primer 3, which is freely available on the internet at the website http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. All primers were manufactured by TAGN (VH Bio Ltd). Primers used for cloning and sequencing equine tryptase and eq.MCP-1 are shown in tables 3.1 and 3.2 respectively. The location and direction of these primers are shown in fig. 3.1.

Equine Tryptase	*Direction*	*Primer Sequence 5'→ 3'*	*Anneal Temp. (°C)*
PT1	sense	TGG CCC TGG CAG GTG AGC CTG AGA	60
PT2	antisense	GGG ACA TAG TGG TGG ATC CA	60
PT3	sense	GAG TCA GTC TGC CAC CTC CGT TTC	55
PT4	sense	CAT TGT GGA AAA CAG CGT TTG TGA	60
PT5	antisense	TGT CTC TGA AAT CTT CAA TGT CCG	55
PT6	antisense	AAC ACA GTG CGC CGC CGT CAG CAC	62
PT7	antisense	TAG GGA GCC CCC GCA GAA GTG TTT	62
PT8	sense	CCA AGA TGC CAA ATC TGC TG	55
PT9	antisense	ATG TTG ACA GGG TCC TGG AG	55
PT10	sense	TCA GAG ACA TCA GGG TGC AG	55
PT11	antisense	GAC GGG GCT CAA GAG TCC TT	55

Table 3.1: Primers used in cloning and sequencing equine tryptase

Eq.MCP-1	*Direction*	*Primer Sequence 5'→ 3'*	*Anneal Temp. (°C)*
PM1	sense	CAC TCC CGT CCC TAC ATG GC	60
PM2	antisense	CAG AAG AGG GCC CCC AGA GTC TCC	60
PM3	sense	CAG ACA CAC TGC AGG AGG TGG	60
PM4	sense	GAG GTG TGC GAA TCC TAC TTC CGC	62
PM5	antisense	CCC AAG GGT GAC ATT CAT TA	55
PM6	antisense	GCA GTG AGC TGC CGT CAG	55
PM7	antisense	GCA CGA GGA CAC CAC CAC AAC TGT	62
PM8	sense	CCA GAG GAG CAC AGG TCT AG	55
PM9	antisense	AGT TCC ACC TCC TGC AGT GT	55
PM10	sense	GGA GAA GAC CCA GCA AGT CA	55
PM11	antisense	GGG TGT TTC TGG ATC AGC TC	55

Table 3.2: Primers used in cloning and sequencing eq.MCP-1

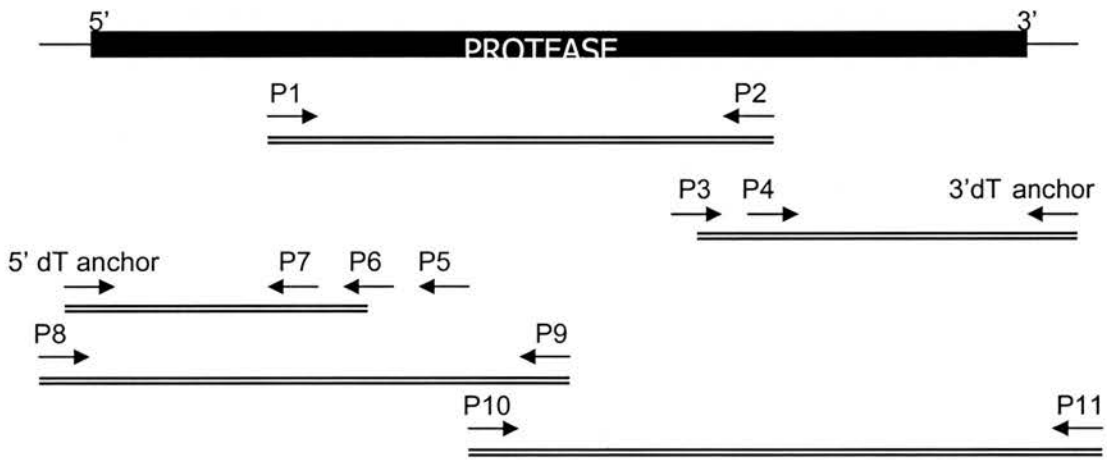

Fig. 3.1: Generic strategy for cloning and sequencing equine tryptase and eq.MCP-1 showing the direction and location of primers used. The heavy black line depicts the protease cDNA sequence whilst the open black lines depict PCR products.

3.3.5. Reverse transcriptase PCR (RT-PCR)

PCR was performed in 10mM Tris-HCl, 1.5mM $MgCl_2$ buffer with 5µl 2mM dNTPs, 10µl 2µM primers and 2.5 units Taq DNA polymerase (Roche). Reactions were performed in a Gene Amp PCR System 2400 (Perkin Elmer) with the following conditions: initial denaturation at 95°C for 5min, followed by 30 cycles of 95°C 40s, annealing temperature 30s (tables 3.1, 3.2), 72°C 1min and a final extension step of 20min at 72°C. PCR products were analysed on 1.2% agarose gels, containing 0.1% ethidium bromide, by electrophoresis at 120-160V for 30min and imaged under UV (Image Station 440 CF, Kodak Digital Science). Gels were then blotted and subjected to subsequent Southern blot analysis with digoxigenin (DIG) labelled probes (section 3.3.13). If clear, distinct bands were obtained on gel electrophoresis, PCR products were sequenced directly (ABI Prism, Durham Sequencing Service, Durham University). If a clear band was not obtained, PCR products were ligated into a vector for subsequent transformation of cells.

3.3.6. Rapid amplification of cDNA ends (RACE)

The 3' and 5' ends of sequences were obtained using a 5'/3' RACE kit (Boehringer Mannheim) as per the manufacturer's recommendations. Briefly, for 3' RACE, first strand cDNA synthesis was initiated at the RNA polyA-tail with the oligo dT-anchor primer. The resulting cDNA was amplified by PCR using P3 and the 3'-dT-anchor primer and then re-amplified using P4 and the 3'anchor. For the 5' end, total RNA was reverse transcribed using P5 and avian myeloblastosis virus reverse transcriptase. The synthesised cDNA was purified using High Pure PCR Product Purification Kit (Roche) and a 3' homopolymeric A-tail added using dATP and terminale transferase, allowing nested PCR using the 5'-dT-anchor primer and primers P6 and P7. The thermal cycle profile used was 94°C for 5min followed by 30 cycles of 95°C 40s, annealing temperature 30s (tables 3.1, 3.2), 72°C 1min, followed by a final extension of 72°C 10min. RACE PCR products were subjected to Southern blot analysis and positive products ligated into a vector.

3.3.7. Ligation of PCR products into Plasmid Vector

PCR products were ligated into the TA cloning vector pCR 2.1 (Invitrogen) according to the manufacturer's directions. Briefly, 2µl of fresh PCR product was incubated overnight at 14°C with 1µl ligation buffer, 2µl pCR 2.1 vector (25ng/µl), 1µl DNA ligase and 4µl sterile water.

3.3.8. Transformation of Competent Cells

The ligated vector was used to transform One Shot INVαF Competent Cells (Invitrogen). A vial of competent cells was thawed on ice and 2µl of ligation reaction added by gentle stirring with a pre-cooled pipette tip. The cells were incubated on ice for 30min and then subjected to a heat shock of 42°C for 30s. The cells were then immediately placed on ice and 250µl room temperature SOC medium (Invitrogen) added. Vials were incubated at 37°C, 225rpm for 1h in a shaking incubator (Gallenkamp, Sanyo) and then 50 and 100µl of the cell suspension plated out onto pre-warmed LB agar containing X-Gal (Promega) and 100µg/ml ampicillin. Agar plates were incubated at 37°C overnight after which small colonies could be seen growing. Colonies of transformed cells were white whereas colonies not transformed by the plasmid were blue. The incubated plates were then stored at 4°C until colony selection.

3.3.9. Isolation of Plasmid DNA

White colonies were transferred using sterile toothpicks into 10ml LB broth containing 100µg/ml ampicillin and incubated overnight at 220rpm, 37°C, after which the LB broth was cloudy from colony growth. Plasmid DNA was isolated from broth medium using Wizard Plus SV Minipreps DNA Purification System (Promega). All steps were performed at room temperature. Cells were pelleted from 3ml broth by centrifugation at 13,000rpm for 5min (Biofuge, Kendro Laboratory Products) and resuspended in 250µl cell resuspension solution. Cells were then lysed by addition of 250µl cell lysis solution and incubated for 5min with 10µl alkaline protease solution to inactivate endonucleases released during cell lysis.

Neutralisation solution (350µl) was then added and the bacterial lysate centrifuged at 13,000rpm for 10min. Plasmid DNA was then bound to a spin column filter by centrifuging the cleared lysate at 13,000rpm for 1min. DNA was washed with 750µl wash solution followed by centrifugation at 13,000rpm for 1min and then a further 250µl of wash solution with centrifugation at 13,000rpm for 2min. Plasmid DNA was then eluted with 100µl nuclease free water (Ambion) and centrifugation at 13,000rpm for 1min.

3.3.10. Restriction Enzyme Analysis of Plasmid DNA

Inserted DNA was cut out of the plasmid using the restriction enzyme Eco R1 (Roche). Plasmid DNA (2µl) was added to 2µl Buffer H (Roche) and 16µl of sterile water. To 10µl of this mixture, 1µl of Eco R1 was added; the other 10µl served as an uncut control. Samples were incubated at 37°C for 2h and then subjected to gel electrophoresis on a 1.2% agarose gel containing 0.1% ethidium bromide at 120V for 45min. After imaging, the gel was blotted for subsequent Southern blot analysis.

3.3.11. Complete Sequence RT-PCR Products

Attempts to amplify the whole cDNA sequence for each proteinase from one PCR reaction were unsuccessful (data not shown). Therefore the cDNA sequence obtained from the three overlapping segments using the protocols above was confirmed by sequencing the whole proteinase in two halves using primers P8 and P9 to amplify the 5' end and P10 and P11 to amplify the 3' end. These products were cloned and sequenced as described above.

3.3.12. DIG-Labelling of Oligonucleotides

Complementary oligonucleotides (oligos) to the DNA sequence of interest were labelled with DIG using the Oligo End Labelling Kit (Roche). The following reaction was set up on ice; 4µl tailing buffer, 4µl cobalt chloride solution, 10µl 100pmol oligonucleotide, 1µl DIG ddUTP solution and 1µl terminale transferase. The reaction was incubated at 37°C for 15min, placed on ice and 2µl stop solution (1µl glycogen

solution and 200µl 0.2mM EDTA) added. The DIG-labelled oligo was then added to 10ml Rapidhyb buffer (Amersham) to be used as a probe for Southern blot analysis of PCR products / clones.

3.3.13. Southern Blot Analysis

Agarose gels of PCR products and restriction digests of cloned products were washed briefly with distilled water and denatured in 1.5M NaCl, 0.5M NaOH twice for 15min. Gels were again washed briefly with distilled water and then neutralised with 1.5M NaCl, 0.5M Tris, 0.001M EDTA twice for 20min. The gel was placed face down on a plastic tray and a piece of nitrocellulose membrane (Amersham) pre-wetted with 1.5M NaCl, 0.5M Tris, 0.001M EDTA placed on top of the gel. Three layers of blotting paper, a thick layer of absorbent paper towels and then a 500g weight were positioned on top of the nitrocellulose membrane. Following overnight capillary transfer of DNA to the nitrocellulose membrane from the gel, the membrane was washed briefly with 6 x SSC (where 1 x SSC is 0.15M NaCl / 0.015M sodium citrate). DNA was fixed to the membrane by 2 UV light cycles of 12s each (Template Tamer, Qbiogene). The size markers were excised and stained by 10min immersion in 5% glacial acetic acid followed by 30min in 0.04% methylene blue (pH 5.2) and final washing with distilled water. The DNA impregnated membrane was pre-hybridised with 10ml Rapidhyb buffer in hybridisation bottles (Techne) in a hybridisation oven (Techne Hybridiser HB-1D) at 40°C for 30min. DIG-labelled oligo probes in Rapidhyb buffer were then hybridised to the membrane for 3h at 40°C. The membrane was washed with 2 x SSC, 0.1% SDS for 2 x 5min, followed by 0.1 x SSC, 0.1% SDS for 2 x 15min. Following a brief rinse with maleic acid buffer (0.15M NaCl, 0.1M maleic acid, pH 7.5), the membrane was incubated with blocking buffer (1% blocking reagent, [Roche] in maleic acid buffer) for 30min followed by anti-digoxigenin-alkaline phosphatase-conjugate (Roche) diluted 1:5000 in blocking buffer for 30min. The membrane was washed twice for 15min with maleic acid buffer, 0.3% Tween 20 (Sigma) and rinsed in 100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5. The reaction was then developed by incubating with

substrate 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium (BCIP / NBT) (Sigma) in the dark until positive bands appeared.

3.3.14. Peptide Mass Fingerprinting

Eq.MCP-1 and tryptase (2μg) previously isolated from equine mastocytoma tissue (Pemberton *et al.*, 2001) were heated to 95°C for 3min with an equal volume of reducing buffer. The reduced proteinases were subject to SDS-PAGE and the gel stained as previously described (section 2.3.1.3). A band was excised for each proteinase, cut into 1mm sections and sent for tryptic peptide mass fingerprinting (Moredun Institute, Midlothian).

3.3.15. Enzyme Kinetics of Equine Tryptase

The enzyme kinetics of equine tryptase with the arginine containing substrate N-(p-Tosyl)-Gly-Pro-Arg p-nitroanilide acetate (GPR, Sigma) and the lysine containing substrate N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate (GPK, Sigma) were assessed to investigate possible preferential activity for one substrate over another.

A single concentration of equine tryptase was incubated at room temperature with a range of concentrations of each substrate (0.25-2.0mM) in 1M glycerol, 0.1M NaCl, 0.1M Tris-HCl, pH 8.0. Analyses were performed in duplicate. The specific activity of equine tryptase in this buffer system has previously been determined with S2366 (Pemberton *et al.*, 2001). Equine tryptase was therefore standardised against S2366 and the final active tryptase concentration in the diluted sample determined to be 3.35×10^{-9}M. The rates of activity for the given substrate concentrations were fitted to a Michaelis-Menten plot using non-linear regression (Graph Pad Prism). Regression analysis determined the kinetic parameters K_m (a measure of the affinity of the enzyme for the substrate) and V_{max} (maximum velocity). V_{max} values ($AUmin^{-1}$) were converted to Ms^{-1} using the extinction coefficient of $15000M^{-1}cm^{-1}$ for paranitroaniline under these conditions (Pemberton *et al.*, 2001). k_{cat} values, a direct measure of the catalytic production under optimum conditions (saturated enzyme), were then calculated from the equation:

$$k_{cat} = \frac{V_{max}}{[\text{Enzyme}]}$$

k_{cat} / K_m ratios were then calculated for each substrate as a measure of overall enzyme efficiency to allow comparison of their suitability as substrates for equine tryptase.

3.3.16. Samples for Expression of Proteinases in Tissues

Tissue samples of liver, colon, skin, bronchus and bronchiole were collected from 3 horses euthansed at R(D)SVS for non-medical reasons. Samples were stored in RNA-Later at 4°C overnight and then at -20°C until further processing. RNA was extracted and reverse transcribed as described in sections 3.3.2 and 3.3.3, respectively.

3.3.17. RT-PCR for Proteinase Expression

RT-PCR was performed as described in section 3.3.5 except that an annealing temperature of 55°C was used over 40 cycles. Each sample was amplified for equine tryptase, eq.MCP-1 and the house-keeping gene β-actin. Primers were designed using Primer 3 and are shown in table 3.3.

Gene	Forward Primer Sequence 5'→ 3'	Reverse Primer Sequence 5'→ 3'	Product Size (bp)
Tryptase	CTC CAG GAC CCT GTC AAC AT	ACG CCA GTG TGG TAT TTC CT	209
Eq.MCP-1	AAG CCA TAC ACC ACC CAG AC	TGC ACA GTC AGT TCC ACC TC	226
β-actin	TGG GCC AGA AGG ACT CAT AC	CTT GAT GTC ACG CAC GAT TT	500

Table 3.3: Primers used in RT-PCR reactions to demonstrate protease expression in various equine tissues.

PCR products were subjected to electrophoresis at 160V, for 30min on a 1.6% agarose gel with 0.1% ethidium bromide and visualised under UV light. Southern blot analysis of PCR products with DIG-labelled probes was performed as described in section 3.3.13.

3.4. Results

3.4.1. Tryptase Sequence

The cDNA sequence for equine tryptase from start to stop codons was 828 base pairs long and is shown in fig. 3.2.

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ATGCCAAATCTGCTGGTGCTGGCACTGGCCCTCCTGGTGAACCTGGGCCACGCGGCCCCTGCCCCAGG
CCAGGCCCTGGAGCGAGAGGGCATCGTAGGAGGACAGGAGGCCTCTGGGAGCAAGTGGCCCTGGCAGG
TGAGCCTGAGAAAGAACACTGAATACTGGAAACACTTCTGCGGGGGCTCCCTAATCCACCCCCAGTGG
GTGCTGACGGCGGCGCACTGTGTTGGACCGGACATTGAAGATTTCAGAGACATCAGGGTGCAGCTGCG
AGAGCAGCACCTCTATTATCGAGACCAGCTGCTGCCCGTCAGCAGGATCCTCCCCCACCCCTACTACT
ACACAGTTGAGAACGGGGCCGACATTGCCCTGCTGGAGCTCCAGGACCCTGTCAACATCTCCAGCCAT
GTCCAGGTGGTCACTCTGCCCCCTGCCTCTGAGACCTTCCCCCCGGGGACGCCGTGCTGGGTGACAGG
CTGGGGCGATGTCGACAATGGAGTCAGTCTGCCACCTCCGTTTCCCCTGAAGGAAGTAAAAGTCCCCA
TTGTGGAAAACAGCGTTTGTGACAGGAAATACCACACTGGCGTGTCCACGGGGGACAACATCCGGATT
GTCCAGGCCGACATGCTGTGTGCAGGGAATAGGAGGCACGACAGCTGCCAGGGCGACTCTGGAGGACC
CCTGGTGTGCAAGGTGAAGGGCACCTGGCTGCAGGCGGGCGTGGTCAGCTGGGCCAACAGCTGTGCTC
AGCCCAACCGGCCGGGCATCTATACCCGTGTCACCTACTACTTGGACTGGATCTACCAGTATGTCCCC
AAGGACTCTTGA
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Fig. 3.2: Equine tryptase cDNA nucleotide sequence from start to stop codon.

This cDNA nucleotide sequence translated into a 275 Aa long protein, consisting of a 30 Aa leader sequence and a 245 Aa mature protein. The cloned sequence corresponded exactly with that determined by N-terminal Aa sequencing of the first 20 residues of tryptase purified from equine mastocytoma tissue (Pemberton *et al.*, 2001). Theoretical trypsin cleavage of the cloned equine tryptase sequence (Peptide Mass, ExPASy) gave 11 peptides in the optimal 600-2000Da range detectable by peptide mass fingerprint MALDI-TOF instrumentation. Peptide mass fingerprint analysis (Mascot, Matrix Science) of equine mastocytoma derived tryptase matched 9 out of these possible 11 peptides, scoring highest compatibility with the above equine tryptase sequence, with a Mascot probability of 0.006 and covering 33% of the mature protein sequence (fig. 3.3).

IVGGQEASGS KWPWQVSLRK NTEYWKHFCG GSLIHPQWVL TAAHCVGPDI
EDFRDIRVQL REQHLYYRDQ LLPVSRILPH PYYYTVENGA DIALLELQDP
VNISSHVQVV TLPPASETFP PGTPCWVTGW GDVDNGVSLP PPFPLKEVKV
PIVENSVCDR KYHTGVSTGD NITRTVQADMT CAGNRRHDSC QGDSGGPLVC
KVKGTWLQAG VVSWANSCAQ PNRPGIYTRV TYYLDWIYQY VPKDS

Fig. 3.3: Coverage map of matched peptides from peptide mass fingerprinting of tryptase isolated from equine mastocytoma tissue to the cloned equine tryptase sequence. Nine out of a possible 11 peptides in the 600-2000Da range matched the sequence (shown in bold red), covering 33% of the mature protein sequence.

The peptide masses produced from the peptide mass fingerprint of the mastocytoma derived tryptase were all within 0.05Da of the peptide masses predicted by trypsin digestion of the cloned sequence (fig. 3.4) enhancing confidence in the assignment. The cloned cDNA nucleotide and Aa sequences were submitted to the GenBank database as equine tryptase (accession number AJ515902).

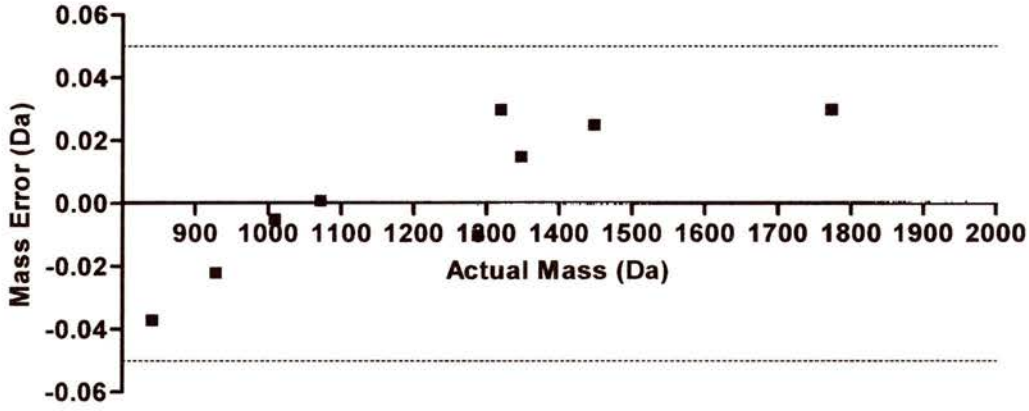

Fig. 3.4: Error between expected tryptic peptide masses for equine tryptase and the observed peptide masses. All observed peptide masses have low errors when compared to their predicted masses (dotted lines represent +0.05 and -0.05).

The cDNA nucleotide sequence for equine tryptase shared greatest identity (81%) with human tryptase-β and 78% identity with sheep tryptase-2. The corresponding Aa sequence shared 77% identity with human tryptase-β and 73% with sheep tryptase-2. Alignment analysis of these proteinase Aa sequences by Clustal W (European Bioinformatics Institute) is shown in fig. 3.5.

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Eq MPNLLVLALALLVNLGHAAPAPGQALEREGIVGGQEASGSKWPWQVSLRKNTEYWKHFCG 60
Hu MLNLLLLALPVLASRAYAAPAPGQALQRVGIVGGQEAPRSKWPWQVSLRVHGPYWMHFCG 60
Sh --MLHLLALALLLSLVSAAPAPGQALQRSGIIGGKEAPGSRWPWQVSLRVRDQYWRHQCG 58
     * :***.:* .   *********:* **:**:**. *:******** .  ** * **

Eq GSLIHPQWVLTAAHCVGPDIEDFRDIRVQLREQHLYYRDQLLPVSRILPHPYYYTVENGA 120
Hu GSLIHPQWVLTAAHCVGPDVKDLAALRVQLREQHLYYQDQLLPVSRIIVHPQFYTAQIGA 120
Sh GSLIHPQWVLTAAHCIGPELQEPSDFRVQLREQHLYYQDRLLPISRVIPHPHYYMVENGA 118
   ***************:**::::   :***********:*:***:**:: ** :* .: **

Eq DIALLELQDPVNISSHVQVVTLPPASETFPPGTPCWVTGWGDVDNGVSLPPPFPLKEVKV 180
Hu DIALLELEEPVNVSSHVHTVTLPPASETFPPGMPCWVTGWGDVDNDERLPPPFPLKQVKV 180
Sh DIALLQLEEPVSISRHVQPVTLPPASETFPPESQCWVTGWGDVDNGRPLPPPYPLKQVKV 178
   *****:*::**.:* **: ***********   **********.  ****:***:***

Eq PIVENSVCDRKYHTGVSTGDNIRIVQADMLCAGNRRHDSCQGDSGGPLVCKVKGTWLQAG 240
Hu PIMENHICDAKYHLGAYTGDDVRIVRDDMLCAGNTRRDSCQGDSGGPLVCKVNGTWLQAG 240
Sh PIVENSVCDWKYHSGLSTDYSVPIVQEDNLCAGDGGRDSCQGDSGGPLVCKVNGTWLQAG 238
   **:** :** *** *  *. .: **: * ****:  :***************:*******

Eq VVSWANSCAQPNRPGIYTRVTYYLDWIYQYVPKDS 275
Hu VVSWGEGCAQPNRPGIYTRVTYYLDWIHHYVPKKP 275
Sh VVSWGDGCAKPNRPGIYTRITSYLDWIHQYVPQEP 273
   ****.:.**:**********:* *****::***:..
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Fig. 3.5: Alignment analysis of Aa residues for equine tryptase (Eq), human tryptase β (Hu) and sheep tryptase 2 (Sh). * denotes that the residues or nucleotides in that column are identical in all sequences in the alignment, : denotes that conserved substitutions have been observed, . denotes that semi-conserved substitutions are observed. The signal / activation peptide is underlined, serine proteinase catalytic triad residues are in red, residues governing primary substrate specificity are in blue, putative glycosylation sites are in green, A-B interface residues are in bold type and A-D interfaces are in bold italics. These A-B / A-D interfaces are the monomeric interfaces of the tetrameric active protease configuration as shown in fig. 3.6.

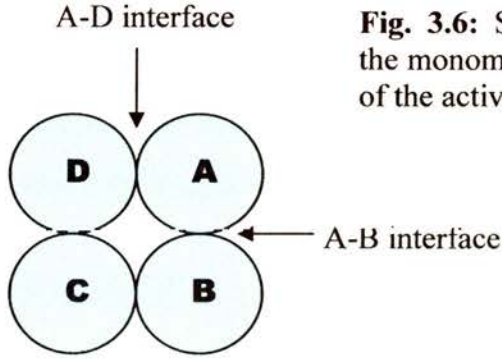

Fig. 3.6: Schematic diagram showing the monomeric interfaces A-B and A-D of the active tryptase tetramer.

The cloned equine tryptase sequence contains residues highly conserved within trypsin-like proteinases, including the activation site region IVGG (residues 31-34) preceded by a 30 Aa leader sequence, WVLTAAHC active site histidine region (residues 68-75), a DIALL active site aspartic acid region (residues 120-124), M-CAG (residues 208-212) downstream of the active site serine and GDSGGP active site serine region (residues 221-226). Potential N-linked carbohydrate binding regions were present at positions 132 and 233. Residues at monomer interfaces, illustrated schematically in fig. 3.6, were identified by alignment with human tryptase-β, where these contact residues have previously been identified (Pereira *et al.*, 1998).

Further sequence observations are made using the archetypal chymotrypsinogen numbering system, which is based upon the alignment of serine proteinase amino acid sequences with chymotrypsinogen. Thus, Ser195 is the catalytic residue of all serine proteinases. Equine tryptase, like all trypsin-like proteinases, contains an aspartate at residue 189 which confers substrate specificity for cleavage C-terminal to arginine or lysine at the P_1 residue (nomenclature of Shechter and Berger [1967]) except when followed by proline (Steitz *et al.*, 1969). Unusually for a trypsin-like proteinase, equine tryptase contains an alanine, rather than a glycine, at residue 216. As this residue forms part of the substrate binding pocket wall (fig. 3.7), this substitution may subtly alter substrate specificity. Enzyme kinetics of equine tryptase for arginine and lysine substrates were therefore investigated (section 3.4.2). Other conserved trypsin-like proteinase features present are the cysteine residues at 191 and 220, the disulphide bond between which acts to support the substrate binding pocket.

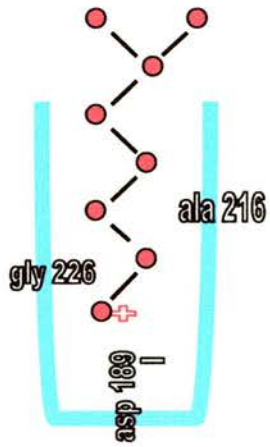

Fig. 3.7: Schematic representation of the substrate binding pocket of equine tryptase. Asp 189 confers a negative charge on the substrate binding pocket attracting side chains with a positive charge e.g. Arg or Lys. The presence of an alanine rather than glycine at residue 216 is highly unusual in trypsin-like proteases.

3.4.2. Enzyme Kinetics of Equine Tryptase

The Michaelis-Menten plot of rates of equine tryptase activity with varying concentrations of the p-nitroanilide substrates GPK and GPR is shown in fig. 3.8. K_m, V_{max}, k_{cat} and k_{cat} / K_m ratios for each substrate are shown in table 3.4.

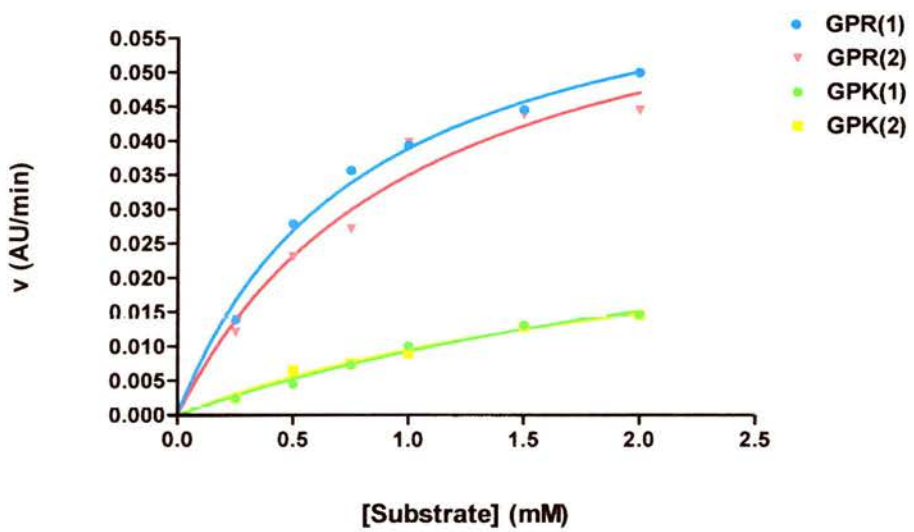

Fig. 3.8: Michaelis-Menten plot of equine tryptase activity rates with varying substrate concentrations of GPK and GPR. The number in parentheses following the substrate name denotes the replicate number.

	GPR(1)	GPR(2)	GPR(av)	GPK(1)	GPK(2)	GPK(av)
K_m (mM)	0.802	1.042	0.922	3.370	2.328	2.849
V_{max} ($AUmin^{-1}$)	0.070	0.072	0.071	0.041	0.032	0.036
k_{cat} (s^{-1})	23.3	23.7	23.5	13.5	10.6	12.0
k_{cat}/K_m ($M^{-1}\ s^{-1}$)	$2.90x10^4$	$2.28x10^4$	$2.59x10^4$	$4.01x10^3$	$4.55x10^3$	$4.28x10^3$

Table 3.4: Substrate profiles for equine tryptase with the substrates GPR and GPK. Numbers in parentheses following the substrate name denotes the replicate number, (av) indicates the average value for that substrate.

The mean k_{cat}/K_m ratio for GPR was six times greater than that of GPK, indicating preferential selectivity of equine tryptase for arginine substrates over lysine substrates.

3.4.3. Putative Eq.MCP-1 Sequence

The cDNA nucleotide sequence of cloned putative eq.MCP-1 was 744 base pairs long and is shown in fig. 3.9 from start to stop codons. This translated into a 247 Aa protein, composed of a 20Aa leader sequence and a 227 Aa catalytic protein, with a molecular weight of 27,464 Da.

```
ATGCGGCCACTCCTGCTTTTGTTAGCGTTTCTTCTGCCTCCTGAGGCTGGGACAGAGGACATCATCGG
AGGACATGAGGCCAAGCCCCACTCCCGTCCCTACATGGCATTGGTTCAATTTCTGTTTGAAGAGATAC
TGCACAGTTGTGGTGGTGTCCTCGTGCGACAGGACATTGTTCTGACGGCAGCTCACTGCTGGGGAAGA
TTAATGAATGTCACCCTTGGGGCCCACAACATCAGGAGGCAGGAGAAGACCCAGCAAGTCATCACTGT
AAGACAAGCCATACACCACCCAGACTATAATCCTAAGAGCTTCTCCAACGACATCATGTTACTAAAGC
TGGGGAGAAGGGCCAAGCTGACTGCAGCTGTGCGGCCCCTCAGCCTGCCCAGGGGCAAGACCCAGGTG
AGGCCCGGAGAGGTGTGCAGTGTGGCTGGCTGGGGGCAACTTGCCCCGAAGGGCAGGTTCCCAGACAC
ACTGCAGGAGGTGGAACTGACTGTGCAGCAGGATGAGGTGTGCGAATCCTACTTCCGCAATTATTTCA
ACAGTACCACTCAGCTGTGTGTAGGGGATCCGAAAGATAAGAAGTCTTCCTTTCAGGGTGACTCTGGG
GGCCCTCTCATCTGTGAGAACGGGCTCCAGGGCATTGTCTCCTATGGACTAGATAACGGGAGTATTCC
ACAGGCCTTCACCAAAGTCTCGAGTTTCCTGCCCTGGATAAAGAAAACCATGAAAAGGCTCTAA
```

Fig. 3.9: cDNA sequence from start to stop codon for the cloned putative eq.MCP-1 protease

However, the first 20 residues of the N-terminal Aa sequence for putative eq.MCP-1 deduced from the cDNA nucleotide sequence differed by two Aas from the

proteinase isolated from equine mastocytoma tissue and assigned the name eq.MCP-1 (Pemberton *et al.*, 2001) (table 3.5). The cloned cDNA and Aa sequences were submitted to GenBank as *putative* equine mast cell proteinase-1 (AJ548473).

Proteinase	N-terminal Aa sequence
Mastocytoma eq.MCP-1	IIGGHEARPHSRPYMAFVQF
Cloned putative eq.MCP-1	IIGGHEAKPHSRPYMALVQF

Table 3.5: N-terminal Aa sequence of mastocytoma derived eq.MCP-1 and cloned putative eq.MCP-1. Differing Aa residues are shown in red in the cloned sequence.

Peptide mass fingerprinting of eq.MCP-1 isolated from equine mastocytoma tissue was performed on two occasions. Fourteen peptide masses matched between these two fingerprint analyses, however neither analysis resulted in a positive match with any known protein sequence from the GenBank database, including the above putative eq.MCP-1 sequence. In fact, only three of these 14 matching peptides corresponded with predicted peptides from the cloned putative eq.MCP-1 sequence (fig. 3.10) suggesting that in fact a similar, but novel, proteinase had been cloned.

IIGGHEAKPH SRPYMALVQF LFEEILHSCG GVLVRQDIVL TAAHCWGRLM NVTLGAHNIR
RQEKTQQVIT VRQAIHHPDY NPKSFSNDIM LLKLGRRAKL TAAVRPLSLP RGKTQVRPGE
VCSVAGWGQL APKGRFPDTL QEVELTVQQD EVCESYFRNY FNSTTQLCVG DPKDKKSSFQ
GDSGGPLICE NGLQGIVSYG LDNGSIPQAF TKVSSFLPWI KKTMKRL

Fig. 3.10: Mature protein Aa sequence for cloned putative eq.MCP-1. Residues in red are the predicted trypsin digested peptides of cloned putative eq.MCP-1 that match peptides from peptide mass fingerprinting of eq.MCP-1 isolated from equine mastocytoma tissue.

The putative eq.MCP-1 cDNA nucleotide sequence shared greatest identity (75%) with the cytotoxic T lymphocyte associated serine esterases human granzyme B and H. The closest related mast cell proteinases were the sheep chymase mast cell proteinase-3 (SMCP-3, 71% identity) and the dual specific (exhibiting both trypsin-like and chymotrypsin-like specificity) sheep mast cell proteinase-1 (SMCP-1, 70% identity). The translated Aa sequence shared 65% identity with human granzyme B,

63% with human granzyme H, 57% with SMCP-3 and 56% with SMCP-1. The alignment analysis of three of these Aa sequences is shown in fig. 3.11.

```
Eq MRPLLLLLAFLLPPEAGTEDIIGGHEAKPHSRPYMALVQFLFEEILHSCGGVLVRQDIVL 60
HG MQPILLLLAFLLLPRADAGEIIGGHEAKPHSRPYMAYLMIWDQKSLKRCGGFLIQDDFVL 60
S3 -MVLFLLLVALLSPAGEAGKIIGGHEAKPHSRPYMAFLQFKISGKSYICGGFLVREDFVL 59
      ::***. ** * . : .**************** : :  .     ***.*:::*:**

Eq TAAHCWGRLMNVTLGAHNIRRQEKTQQVITVRQAIHHPDYNPKSFSNDIMLLKLGRRAKL 120
HG TAAHCWGSSINVTLGAHNIKEQEPTQQFIPVKRPIPHPAYNPKNFSNDIMLLQLERKAKR 120
S3 TAAHCLGSSINVTLGAHTITDQERTQQVIQVRRAIPHPDYNDETCANDIMLLQLTRKAEM 119
   ***** *  :*******.*  ** ***.* *::.* ** ** :. :******:* *:*:

Eq TAAVRPLSLPRGKTQVRPGEVCSVAGWGQLAPKGRFPDTLQEVELTVQQDEVCESYFRNY 180
HG TRAVQPLRLPSNKAQVKPGQTCSVAGWGQTAPLGKHSHTLQEVKMTVQEDRKCESDLRHY 180
S3 TDAVSLINLPRSLEKVKPGMMCSVAGWGQLGVNMPSADKLQEVDLEVQREEKCIARFKDY 179
   * **  : ** .  :*:**  ******** .     ...****.: **.:. * : ::.*

Eq FNSTTQLCVGDPKDKKSSFQGDSGGPLICENGLQGIVSYGLDNGSIPQAFTKVSSFLPWI 240
HG YDSTIELCVGDPEIKKTSFKGDSGGPLVCNKVAQGIVSYGRNNGMPPRACTKVSSFVHWI 240
S3 IP-VTQICAGDPSKRKDSFLGDSGGPLVCDGVAQGIVSYGKDDGTTPNVYTRISSFLSWI 238
     . ::*.***. :* ** *******:*:   ******* ::*  *.. *::***: **

Eq KKTMKRL------ 247
HG KKTMKRY------ 247
S3 QRTMRQYKNQGSA 251
   ::**::
```

Fig. 3.11: Aa alignment analysis of putative eq.MCP-1 (Eq), human granzyme B (HG) and sheep mast cell protease 3 (S3). * denotes that the residues or nucleotides in that column are identical in all sequences in the alignment, : denotes that conserved substitutions have been observed, . denotes that semi-conserved substitutions are observed. The signal / activation peptide is underlined, serine proteinase catalytic triad residues are in red, residues governing primary substrate specificity are in blue, putative glycosylation sites are shown in green, residues in bold are the predicted trypsin digested peptides of cloned putative eq.MCP-1 that match peptides from peptide mass finger printing of eq.MCP-1 isolated from equine mastocytoma tissue.

Further classifications are made using the chymotrypsinogen numbering system. The active site H, D, S residues are located at positions 35, 79 and 174, respectively. The uncharged glutamine residue at position 226 is expected to confer chymase substrate specificity with preference for uncharged, aromatic substrates (Zamolodchikova *et al.*, 2003). Other conserved features specific to non-trypsin-like proteinases present within the putative eq.MCP-1 sequence include the Cys136-Cys201 disulphide bond and the conserved N-terminal sequence (IIGG residues 16-19 and PHSRPYMA

residues 24-31). Importantly, the putative eq.MCP-1 Aa sequence displays the main structural characteristic of graspases; the absence of the active site double cysteine bond between residues 191 and 220. The latter is highly conserved among other serine proteinases (Zamolodchikova *et al.*, 2003).

3.4.4. Expression of Proteinases in Equine Tissues

Both equine tryptase and putative eq.MCP-1 were expressed in all tissues analysed; bronchus, bronchiole, liver, skin and colon (fig. 3.12). Some genomic DNA contamination was evident in some samples. Southern blot analyses of gels with DIG-labelled oligos were positive.

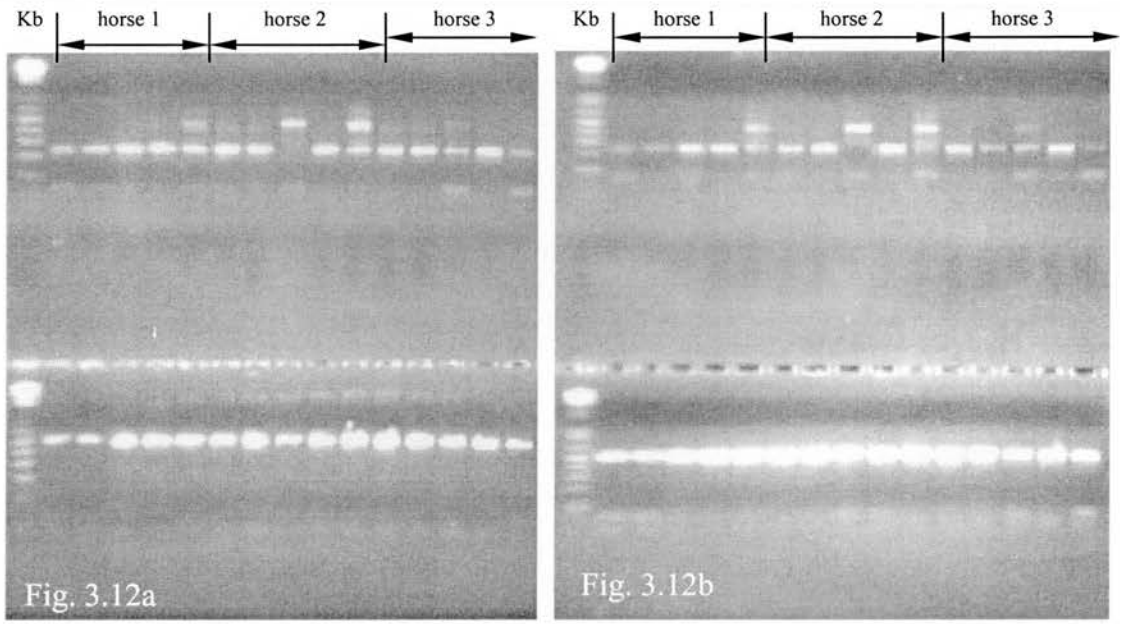

Fig. 3.12: PCR products of tryptase (a) and eq.MCP-1 (b) expression in equine tissues run out on a 1.6% agarose gel with 0.1% ethidium bromide at 160V for 30min. The top row of PCR products are protease fragments (209bp tryptase, 226bp eq.MCP-1), the bottom row of products are β-actin fragments (500bp). Samples are ordered: bronchiole, bronchus, liver, colon, skin for each horse in turn. Kb = Kilobase pair markers (25-1000bp).

3.5. Discussion

3.5.1. Cloning and Sequencing of Equine Tryptase

This is the first reported cDNA nucleotide sequence for equine tryptase. The cloned equine tryptase Aa sequence was the same length (245 Aa mature protein) and shared >70% identity with other mammalian tryptases. The N-terminus corresponded exactly with that determined by N-terminal Aa sequencing of the first 20 residues of equine tryptase isolated from equine mastocytoma tissue. Furthermore, results of peptide mass fingerprinting of mastocytoma derived tryptase corresponded well with the cloned Aa sequence. We are therefore confident that equine tryptase was cloned and sequenced.

Many features of the cloned equine tryptase sequence were characteristic of trypsin-like proteinases, including the aspartate 189 residue governing substrate specificity, the cysteine residues at 191 and 220 and the highly conserved tryptase sequence regions of the activation site region and active site residues (Furie *et al.*, 1982). The 30 Aa leader sequence is followed immediately by the 245 Aa catalytic proteinase sequence without an intermediate activation peptide which is also typical of serine proteinases, and tryptase in particular (Miller *et al.*, 1989). Mast cell granules contain active tryptase without this 30 Aa leader peptide and therefore roles for directing the proteinase into the granules or in formation of the active tetramer from the individual monomers have been suggested for the leader sequence (Miller *et al.*, 1989; Caughey, 1991).

In addition to the importance of residue 189 conferring substrate specificity, the backbone conformation around residue 216 is critical in determining serine proteinase specificity (Perona *et al.*, 1995). The presence of an alanine residue at 216 in equine tryptase is highly unusual among tryptases. The only other reported tryptase which does not have a glycine residue at position 216 is human tryptase-α, which has an aspartate substitution (Miller *et al.*, 1989). Residues 216 and 226 form part of the walls of the substrate binding pocket and are usually both glycines in trypsin-like proteinases. The aspartate 189 residue, these glycine residues and the

double cysteine bond between residues 191 and 220 are believed to give a rigid conformational shape to the substrate binding pocket with the small glycine residues allowing access of bulky arginine and lysine side chains to the negatively charged aspartate at the bottom of the pocket. Alanine contains a methyl (CH_3) side chain, which is larger than the glycine side chain (H), and will therefore project further into the substrate pocket. This substitution may therefore influence substrate binding pocket conformation and consequently substrate specificity in a subtle way. Indeed, kinetic profiles of equine tryptase with arginine and lysine substrates (GPR and GPK) revealed a six-fold preferential selectivity for the arginine substrate. In contrast, trypsin and other tryptases generally show equivalent enzyme kinetics with these same two substrates (Huang *et al.*, 1997) which are identical except for arginine or lysine at the P_1 residue, indicating that this preferential selectivity must be associated with these P_1 residues. Increased specificity for arginine substrates is somewhat counter-intuitive as arginine bears a bulkier side chain than lysine. However, this is consistent with the genetically engineered substitution of alanine for glycine at residue 216 in trypsin, which also resulted in increased selectivity for arginine substrates (Craik *et al.*, 1987). Other naturally occurring substitutions in this area include that of an aspartate for glycine at residue 216 in human tryptase-α which increases tetramer stability compared with human tryptase-β (Selwood *et al.*, 2002) but alters substrate specificity such that it is unable to cleave fibrinogen (Huang *et al.*, 1999). However, as human tryptase-α is the predominant tryptase found in peripheral blood (Schwartz *et al.*, 1995), this may be a biologically advantageous mutation. The biological significance of the alanine 216 substitution in equine tryptase is unknown but may be important with regard to cleavage of fibrinogen-β chains. Human tryptase cleaves the human fibrinogen-β chain at the P_1 residue lysine-21 (Thomas *et al.*, 1998). Currently, only a 19 residue fragment of the Aa sequence encoding equine fibrinogen-β chain has been determined and therefore it is unknown if this lysine-21 P_1 residue also exists in the equine fibrinogen-β sequence. However, if this lysine residue is present, the alanine 216 substitution could have a significant biological effect restricting fibrinogenolysis *in vivo*.

Tryptase is enzymatically active as a heparin-stabilised tetramer of 120-140 kDa consisting of four tryptase monomer subunits of 31-34kDa in a ring-like structure with a central pore (Pereira *et al.*, 1998). Each monomer contacts neighbouring monomers at two sites (demonstrated as A-B and A-D interfaces in fig. 3.6) and has an active site directed towards the oval central pore (Pereira *et al.*, 1998). This protected location restricts access to the active site and explains the inherent resistance of tryptase to endogenous proteinase inhibitors. Instead of regulation via inhibitors, tryptase activity is thought to be restricted *in vivo* by loss of stabilisation by heparin, or other acidic polysaccharides, from the tetramer such that it dissociates into inactive monomers (Schwartz and Bradford, 1986; Addington and Johnson, 1996). Examples of this are lactoferrin and myeloperoxidase, neutrophil granule proteins which scavenge heparin from tryptase, leading to loss of activity (He *et al.*, 2003). Therefore, interestingly, the inflammatory neutrophil influx into the airway lumen in equine heaves may perform an important role in regulating tryptase activity.

3.5.2. Cloning and Sequencing of Putative Eq.MCP-1

The deduced Aa sequence for putative eq.MCP-1 shared greatest identity (65%) with the lymphocyte derived proteinases, human granzymes B and H, whereas the closest related mast cell specific proteinases shared <60% identity. The N-terminal Aa sequence of eq.MCP-1 extracted from mastocytoma tissue also shared greatest identity with the chymase human granzyme H (Pemberton *et al.*, 2001). However, the N-terminal Aa sequence of this mastocytoma derived eq.MCP-1 differed from that of cloned putative eq.MCP-1 by 2 Aas. The peptide masses predicted by trypsin digestion of the cloned Aa sequence also corresponded poorly with the peptide mass fingerprint of mastocytoma derived eq.MCP-1. Furthermore, a search of all known sequence databases did not result in a positive match for the mastocytoma eq.MCP-1 peptide mass fingerprint. It therefore appears likely that a similar, but novel, proteinase was cloned and sequenced rather than eq.MCP-1. Mast cells of many mammalian species express multiple closely-related chymases (McAleese *et al.*, 1998; Miller and Pemberton, 2002) and therefore the cloned sequence may correspond to another equine mast cell chymase. As a mixed cell population was

used for RNA extraction, it is also possible that the cellular origin of the cloned proteinase is the lymphocyte which as discussed above, may also express chymotrypsin-like proteinases. *In situ* hybridisation would be necessary to confirm the cellular location of this novel proteinase. An alternative explanation for this apparently novel proteinase may be that the deduced eq.MCP-1 sequence does not actually represent a gene product as it could only be constructed from two separate, albeit overlapping, sequences.

It is helpful to consider a recent re-classification of the chymotrypsin family of serine proteinases, which has divided members into the 'trypsin-like' group and the 'graspase' group based upon the presence or absence, respectively, of the disulphide bond between cysteine residues 191 and 220 (Zamolodchikova *et al.*, 2003). Accordingly, by lacking this bond, putative eq.MCP-1 is considered to be a graspase. The cysteine 191-220 disulphide bond is believed to support the architecture of the substrate binding pocket and consequently is thought to be highly important for proteinase activity. Although the loss of this bond from trypsin and chymotrypsin dramatically decreases their enzymic activity and the specificity of trypsin (Varallyay *et al.*, 1997), graspases are active proteinases and display a wider range of specificities than trypsin-like enzymes. However, the residue which contributes most influence over substrate specificity in graspases is residue 226, not 189. The putative eq.MCP-1 sequence also contains other highly conserved graspase features such as the Cys136-Cys201 disulphide bond and the residues 16-19 and 24-31 in the N-terminus.

Graspases are further divided according to the nature of residue 226 into chymozymes (uncharged residue), duozymes (aspartate or glutamine) or aspartases (arginine) (Zamolodchikova *et al.*, 2003). Therefore the presence of an uncharged glutamine residue at position 226 in putative eq.MCP-1 classifies this proteinase as a chymozyme, displaying expected chymotrypsin-like specificity for hydrophobic substrates. Similarly, mastocytoma derived eq.MCP-1 was found to cleave the thioester chymotrypsin substrate FLF-SBzl but not the tryptase substrate S2366 (Pemberton *et al.*, 2001). However substrate specificity of this mastocytoma derived

eq.MCP-1 was found to be very restricted, hydrolyzing only one of four peptide substrates readily cleaved by other chymases (Pemberton *et al.*, 2001). The chymozyme human granzyme H, which has the highest identity with putative eq.MCP-1, also shows highly restricted specificity, cleaving only FLF-SBzl (Edwards *et al.*, 1999). Of the other proteinases that shared high identity with putative eq.MCP-1, only SMCP-3 is an apparent chymozyme by this classification, whilst human granzyme B is an aspartase and SMCP-1 is a duozyme.

Chymotryspin-like mast cell proteinases had previously been classified into α- and β-chymases according to their ability to convert angiotensin I to angiotensin II (Chandrasekharan *et al.*, 1996). Homologous mammalian α-chymases, which can catalyse this conversion, have been identified in man and dog, whereas β-chymases, which do not necessarily possess angiotensin forming ability, are expressed by rodent and ruminant mast cells (Miller and Pemberton, 2002). Eq.MCP-1 isolated from mastocytoma tissue was only able to hydrolyse angiotensin I very slowly and therefore is most likely to be a β-chymase (Pemberton *et al.*, 2001). The distinction between α- and β- chymases is believed to be due to the residues in the extended substrate binding site determining specificity but the precise molecular basis for this has not been deduced (Chandrasekharan *et al.*, 1996).

3.5.3. Proteinase Expression in Equine Tissues

Mast cell proteinase expression in equine tissues has not previously been reported. All tissues analysed (bronchus, bronchiole, colon, liver and skin) were found to express equine tryptase and putative eq.MCP-1. Quantitative analysis was not undertaken and therefore relative tissue expression can not be assessed. Pemberton *et al.* (2001) likewise identified equine tryptase and eq.MCP-1 positive mast cells in equine skin, duodenum and lung with immunohistochemistry. However, due to the uncertain identity of putative eq.MCP-1, immunohistochemically positive cells using antibodies directed against eq.MCP-1 isolated from mastocytoma tissue may represent a different population of cells than those expressing putative eq.MCP-1. A polyclonal antibody was used to identify eq.MCP-1 positive cells and therefore it

may also have recognised other similar mast cell chymase proteins. Sheep tryptase, which shares 73% identity with equine tryptase, is also widely expressed in ovine tissues (Pemberton *et al.*, 2000). In contrast, SMCP-1, which shares 56% identity with putative eq.MCP-1, is less abundantly expressed in normal ovine tissues (Pemberton *et al.*, 2000).

3.6. Conclusion

Equine tryptase was cloned and sequenced and found to be widely expressed in equine tissues. Uniquely among trypsin-like proteinases, equine tryptase has an alanine substitution at residue 216 which confers increased specificity for arginine residues. The biological significance of this substitution *in vivo* remains to be elucidated; however it may restrict fibrinogenolysis. Molecular probes subsequently designed from this sequence were used to investigate mRNA transcripts in the lungs of control and heaves affected horses (Chapter 5). Whilst attempts were made to clone eq.MCP-1, peptide mass fingerprinting of mastocytoma derived eq.MCP-1 indicated that a similar, but novel, chymase had been sequenced. As this cloned proteinase shared strongest identity with lymphocyte derived human granzyme B, a mast cell origin for the cloned chymase could not definitively be determined. This novel chymase was also widely expressed in equine tissues.

Chapter 4: Mast Cell Proteinase Concentration and Expression in BALF from Control and Heaves Susceptible Horses

4.1. Summary

ELISAs were developed and optimised in order to determine concentrations of equine tryptase and eq.MCP-1 in BALF from controls (n=22), heaves horses during clinical exacerbation (n=21), heaves horses in remission (n=13) and horses with other pulmonary disease (n=7). Chromogenic assays were performed to determine BALF tryptase activity. BALF histamine concentrations were measured using a commercial ELISA. Immunolabelling of BALF cytospins was performed to investigate proteinase expression of BALF mast cells in control (n=8) and heaves (n=5) horses.

Clinical heaves horses had significantly increased BALF tryptase concentrations compared to controls and heaves horses in remission. Horses with other pulmonary diseases also had significantly elevated BALF tryptase concentrations compared to controls. BALF from clinical heaves horses also had greater tryptase activity compared to remission horses. Very low eq.MCP-1 concentrations were measured in BALF from all horses. BALF histamine concentrations were not significantly different among groups, which was considered, at least in part, to be the result of histamine degradation in archived samples. BALF mast cells were predominantly tryptase positive with only occasional eq.MCP-1 positive cells observed. There was no significant difference in proteinase content of BALF mast cells between control and heaves horses.

This study demonstrates mast cell degranulation and tryptase release into the pulmonary airspace following challenge of heaves susceptible horses suggesting that mast cell mediators may play a role in the pulmonary inflammatory response of equine heaves.

4.2. Introduction

Mast cell mediators are regarded as playing a key role in the airway inflammation and remodelling which occurs in response to inhaled allergens and irritants in human asthma (Holgate, 2000), despite mast cells constituting only a small proportion of the total BALF cell population in human asthmatics, as they do in heaves horses. Consequently, BALF tryptase and histamine concentrations have been measured as indicators of mast cell degranulation and are significantly elevated in symptomatic asthmatics compared to control subjects (Casale *et al.,* 1987a; Wenzel *et al.,* 1988; Chan-Yeung *et al.,* 1989; Broide *et al.,* 1990; Broide *et al.,* 1991; Jarjour *et al.,* 1991; Bousquet *et al.,* 1991).

Much prior research investigating equine heaves has focused upon the neutrophil due to the influx of large numbers of neutrophils into the airway lumen during exacerbation of disease. However, there is now a growing body of evidence to suggest that mast cells may play an important role in the pathogenesis of equine heaves. This includes elevated BALF IgE to common fungal allergens (Halliwell *et al.,* 1993; Schmallenbach *et al.,* 1998), increased PELF histamine concentrations 5h following challenge of heaves susceptible horses (McGorum *et al.,* 1993c) and increased numbers of bronchiolar mast cells in horses with chronic bronchiolitis (Winder and von Fellenberg, 1988).

The aim of this study was therefore to: (a) determine mast cell proteinase concentrations and activity and histamine concentrations in BALF from control and heaves susceptible horses during disease exacerbation and remission, and (b) investigate proteinase expression of BALF mast cells in control and heaves horses.

4.3. Materials and Methods

4.3.1. Subjects and Collection of BALF for Determination of BALF Mast Cell Proteinase and Histamine Concentrations

Samples utilised comprised those collected from clinical cases at the Royal (Dick) School of Veterinary Studies (RD[S]VS) during routine clinical investigation procedures and archived samples which had been collected during 2 previous studies. The status of horses from which BALF samples were collected is shown in table 4.1.

Status	No. samples
Control	12
Challenged control	10
Clinical heaves	21
Heaves remission	13
Other pulmonary diseases	7
Total	63

Table 4.1: Status of horses from which BALF samples were collected for determination of BALF mast cell proteinase and histamine concentrations.

BALF samples were obtained from three groups of subjects as follows:

4.3.1.1. Horses Undergoing Bronchoalveolar Lavage (BAL) at R(D)SVS

BALF samples (n=24) were collected from horses referred to R(D)SVS for investigation of respiratory disease or poor performance. Following sedation with 30μg/kg romifidine (Sedivet, Boehringer Ingelheim Ltd.) and 15μg/kg butorphanol (Torbugesic, Willow Francis Veterinary) and restraint with a nose twitch, the endoscope (Olympus CF Type 200HL, 1.7m working length, Olympus Optical Co. Ltd.) was introduced via the nares and rima glottidis into the trachea and then advanced until wedged in the right accessory lobe. Following instillation of 300ml 0.9% saline (Ivex) (room temperature) and then approximately 30ml air, BALF was retrieved manually using gentle suction with 60ml syringes. Attempts were made to reduce dwell time of lavage fluid to minimise exchange of molecules between the airspace and pulmonary circulation (Marcy *et al.*, 1987). BALF supernatant was

harvested by centrifugation (400g, 10min) and stored at -70°C until further processing (<6mths).

Three further samples were obtained from control horses that were lavaged immediately post mortem following euthanasia for non-pulmonary reasons. A BAL catheter (240cm length, 10mm external diameter; Irish Equine Centre) was passed blindly into the lungs per nasum until wedged. Thereafter, BAL was performed as described above.

The disease status of these horses was defined by the history (including husbandry practices) and BALF cytology. A history of clinical signs suggestive of heaves following exposure to hay / straw and a differential neutrophil count >5% with normal neutrophil morphology was taken as indicative of clinical heaves (hereafter termed 'heaves') (Dixon *et al.*, 1995b). Horses with no history of coughing or nasal discharge and normal BALF cytology were classified as controls. Horses with normal BALF cytology following prior diagnosis and treatment of heaves were classified as heaves horses in remission (hereafter termed 'remission'). Cases which did not fit into one of these 3 groups and had a mixed inflammatory pattern on BALF cytology were given the status of other pulmonary disease, hereafter termed 'other disease'.

The 24 samples collected from the R(D)SVS hospital referred cases were comprised of 6 controls, 5 heaves, 6 remission and 7 other disease (viral disease n=4, eosinophilic pulmonary disease n=2, inflammatory airway disease n=1). The median and range of ages and sex distribution for these groups is shown in table 4.2. This sample population is hereafter termed R(D)SVS hospital.

Group	**No. horses**	**Median (range) age (years)**	**Sex**
Control	6	7 (4-18)	1 F, 5 MN
Heaves	5	12 (4-20)	2 F, 2 MN, 1 M
Remission	6	12 (10-30)	2 F, 4 MN
Other pulmonary disease	7	7 (6-12)	7 MN

Table 4.2: Age and sex distribution of horses in R(D)SVS hospital population. Key: F female, MN gelding, S stallion.

4.3.1.2. Archived Samples from Natural Hay / Straw Challenge of Control or Heaves Susceptible Horses at R(D)SVS

In a former study, horses previously classified by natural hay / straw challenge as controls (n=3) or heaves susceptible (n=5) horses, were exposed for 5h to a challenge environment consisting of a small (3.7m x 3.7m), poorly ventilated stable with the doors and air vents closed, a deep litter straw bed and fed dusty hay with visible mould growth. The median (range) total and respirable dust concentrations in this challenge environment were 1.5 (0.5-1.9) and 0.2 (0.1-0.3) mg/m^3 respectively. This hay / straw challenge has previously been shown to induce heaves in susceptible horses only (McGorum *et al.*, 1993d). BALF samples and supernatant were collected as described above at 6h and / or 24h following initiation of challenge and stored at -70°C for 2-4 years prior to further processing.

There were 4 samples from the 3 control horses (all mares) with a median (range) age of 4y (4-6y). Nine samples were from the 5 heaves susceptible ponies (3 mares, 2 geldings) with a median age 14y (7-24y). This sample population is hereafter termed R(D)SVS archive.

4.3.1.3. Archived Samples from Natural Hay / Straw Challenge of Control or Heaves Susceptible Horses at the Animal Health Trust (AHT), Newmarket

Horses categorised, as described above, into control (n=6) or heaves susceptible (n=7) horses underwent a 24h natural hay / straw challenge to induce disease in susceptible animals in a model similar to that described above. Stable dimensions were 3.6m x 5.6m with an airspace volume of $72m^3$. Total and respirable dust concentrations were not performed for this challenge model however mould species analysis was available. Hay and straw used in this challenge contained 286 x 10^8/ml particulates (<60μm diameter) and 200cfu/g *Thermophilic actinomycetes* and 69cfu/g *Aspergillus fumigatus* in the hay and straw, respectively. BAL was performed 7 days prior to challenge (baseline) and immediately following cessation of challenge with an instillation volume of 200ml 0.9% saline, pre-warmed to 37°C. Supernatant was harvested (800g, 10min, 4°C) and frozen at -70°C for <1 year.

There was 1 mare and 12 geldings with the median (range) control age of 7y (5-15 years) and median heaves susceptible age of 15y (8-23 years). This sample population is hereafter termed AHT archive.

4.3.2. Processing of BALF

4.3.2.1. Preparation of Cytospins and Differential Cell Counts (DCC)

Cytospins were prepared in duplicate from R(D)SVS hospital samples by cytocentrifugation of 100µl BALF at 300g, for 3min (Shandon Cytospin 3). Cytospins were air dried and then stained with Leishmans stain (Fisher Scientific). DCC of 500 cells were performed on each cytospin and mean counts calculated for each horse from duplicate slides. Cytospins and DCC had previously been performed in an identical manner for R(D)SVS archive samples. DCC were obtained from the AHT (Shandon Cytospin 3, minimum 200 cells, stained with haematoxylin and eosin) for AHT archive samples.

4.3.2.2. Concentration of BALF Samples

As preliminary trials showed that unconcentrated BALF proteinase concentrations were very low, frozen BALF supernatant was thawed on ice and concentrated using a 10kDa centrifugal filter device (Centricon, Millipore) at 5000g for 90min. This technique has been used previously in studies measuring tryptase in human BALF and nasal lavage fluid (Castells and Schwartz, 1988; Wenzel *et al.,* 1988; Broide *et al.,* 1990; Sedgwick *et al.,* 1991; Jarjour *et al.,* 1991). The mass of supernatant was measured pre- and post-centrifugation to enable calculation of the concentration factor. Proteinases, having a molecular weight greater than 10kDa, were retained in the upper reservoir, whilst histamine passed through into the filtrate. The mean (range) concentration factor was 2.74 (1.31-7.73).

4.3.2.3. Determination of Tryptase and Eq.MCP-1 Concentration by ELISA

For all ELISAs, primary coating antibodies (1µg/ml) in carbonate buffer (pH 9.6) were applied (50µl/well) to plastic 96 well plates (Immulon 96W, Dynatech M 129B, Thermo Life Sciences) (hereafter termed ELISA plates) and incubated overnight at 4°C. All further dilutions used ELISA buffer (4% bovine serum albumin [Sigma] / PBS / 0.05% Tween 20 [Sigma]) unless otherwise stated. All incubation steps were for 1h at 37°C unless otherwise stated, and were followed by washing which consisted of six washes with 0.9% saline containing 0.05% Tween 20, followed by rigorous tapping of the plate onto absorbent paper to dry wells. Analyses were performed in duplicate and all steps used a final volume of 50µl/well. Following incubation with the secondary antibody, all ELISAs were developed using streptavidin-horse radish peroxidase conjugate (S-HRP) (Amersham Biosciences, and latterly Sigma) for 30 min (Amersham Biosciences, 1:5000) or 60 min (Sigma, 1:1000) followed by TMB substrate (Insight Biotechnology) which caused a blue colouration of positive samples. Following adequate colour development, the reaction was stopped with 0.18M H_2SO_4. Absorbance of samples was read in a microplate reader (Bio-Rad Model 550, Bio-Rad Laboratories) at 450nm. The standard curve was generated using a linear curve fit and sample concentrations calculated using Microplate Manager 4.0 software (Bio-Rad Laboratories) and corrected for concentration factor.

4.3.2.4. Development of Proteinase ELISAs

To test antibody specificity for equine tryptase, ELISA plates were coated with 1µg/ml equine tryptase (previously extracted from equine mastocytoma tissue as described by Pemberton *et al.* [2001]) overnight at 4°C and then incubated with 1/1000 or 1/5000 mouse monoclonal anti-human tryptase-biotin (clone AA5, Promega) or 1µg/ml or 0.2µg/ml polyclonal rabbit anti-equine tryptase followed by biotinylated goat anti-rabbit IgG at 1/400 or 1/2000.

Following successful recognition of equine tryptase by both these antibodies, a sandwich ELISA was developed. ELISA plates were coated with 1µg/ml polyclonal

rabbit anti-equine tryptase and then a dilution series of equine tryptase (1-100ng/ml) in ELISA buffer applied. Mouse monoclonal anti-human tryptase-biotin at 1/1000 or 1/3000 dilution was then used as the secondary antibody. This ELISA failed to detect tryptase and therefore an alternative sandwich ELISA was investigated using mouse monoclonal anti-human tryptase (clone AA1, Novocastra) as the coating antibody at 1/100, 1/500 or 1/1000 dilution and polyclonal rabbit anti-equine tryptase as the secondary antibody (0.5µg/ml) followed by biotinylated goat anti-rabbit IgG at 1/2000 dilution.

Following lack of recognition of tryptase by mouse monoclonal anti-human tryptase (clone AA1), biotinylated rabbit polyclonal anti-equine tryptase was tested for specificity against equine tryptase using 0.2, 0.5 and 1µg/ml dilutions on wells coated with 1-100ng/ml tryptase. Rabbit polyclonal anti-eq.MCP-1 and biotinylated rabbit polyclonal anti-eq.MCP-1 were likewise investigated for specificity against eq.MCP-1 coated wells. As these antibodies successfully recognised their respective target proteinases, sandwich ELISAs using rabbit polyclonal anti-equine tryptase / anti-eq.MCP-1 as the coating antibody and biotinylated rabbit polyclonal anti-equine tryptase / anti-eq.MCP-1 as the secondary antibody were tested. A range of tryptase / eq.MCP-1 concentrations were used on the ELISAs to find the upper limit of linearity of the standard curves.

4.3.2.5. Final Proteinase ELISA

ELISA plates were coated with 1µg/ml polyclonal rabbit anti-equine tryptase / eq.MCP-1. Standards (0.25-10ng/ml tryptase / 0.25-20ng/ml eq.MCP-1 in ELISA buffer), blanks and samples were applied. Wells containing ELISA buffer alone were used as blanks. Biotinylated rabbit polyclonal anti-equine tryptase / eq.MCP-1 (1µg/ml) were applied as secondary antibodies.

4.3.3. Validation of Mast cell Proteinase ELISAs

4.3.3.1. Control ELISA Experiment

ELISAs were performed with a positive control of proteinase at mid standard curve concentration (5ng/ml tryptase, 10ng/ml eq.MCP-1) plus a series of negative controls each omitting one component of the ELISA to ensure that none of the reagents used were responsible for non-specific colour development.

4.3.3.2. Spiked BALF ELISA

To validate the proteinase concentrations determined using the ELISA, unconcentrated BALF and BALF spiked with 1000, 500, 100, 50 and 25ng/ml tryptase and 1000, 500 and 100ng/ml eq.MCP-1 were applied to the ELISA. Serial dilutions of spiked BALF were assessed to ensure that at least one reading fell within the standard curve.

4.3.3.3. Reduction of Background Colouration in Tryptase ELISA

When serial dilutions of unconcentrated or spiked BALF were analysed by the tryptase ELISA, an unacceptably high level of background colouration was observed. In an attempt to determine the nature of this background colouration, a series of experiments was undertaken.

Firstly, a blocking step of incubating plates with ELISA buffer for 30min was employed, before addition of the secondary antibody, to reduce any non-specific binding.

As BALF mucus was considered a possible source of interference in the ELISA, 0.5mg/ml dithiothreitol (DTT, Sigma) was added to serial dilutions of unconcentrated BALF and of BALF spiked with 100ng/ml tryptase, to reduce disulphide bonds quantitatively and maintain monothiols in the reduced state. As BALF from heaves affected horses contains more mucus than that from control horses (Dixon *et al.*, 1995b), BALF from both groups of horses was used. Samples

with DTT were read against a standard curve with DTT added, because DTT may reduce the slope of the ELISA curve (*Mark Buckley, personal communication*).

In a further attempt to minimise the potential effect of insoluble mucus on the ELISA, BALF samples from control and heaves horses (previously concentrated by Centricon YM10 filters) were subject to high speed centrifugation at 20,000g, for 10min, 4°C and filtration through a 0.45μm filter (Millex-HV Durapore filters, Millipore Corporation) prior to testing on the ELISA.

4.3.3.4. Heparin-Agarose Tryptase ELISA

To ascertain that the tryptase ELISA was measuring tryptase specifically, an ELISA using concentrated BALF supernatant with and without addition of heparin-agarose, which binds tryptase, was investigated. A suspension of heparin-agarose beads in saline (pH 6.0) was added to BALF in a 1:1 dilution, mixed briefly and then sedimented by centrifugation at 1000rpm, 1min. BALF diluted 2:1 with saline was used as a negative control, this being equivalent to the net volume of liquid added to samples with the agarose beads. Positive controls consisted of 150μl saline spiked with 10μl of 1000ng/ml tryptase (resultant concentration of 66.7ng/ml) and saline spiked with tryptase diluted 1:1 with heparin agarose.

4.3.3.5. Intra- and Inter-assay Coefficients of Variation (CV) for ELISA

To determine intra-assay variability, measurement of proteinase concentration was repeated 8 times within the same plate. Inter-assay variability was determined by running the same sample on different ELISA plates on 5 separate occasions. For tryptase CV experiments, one sample from a control horse and one sample from a heaves horse were chosen. For eq.MCP-1, only one sample was used as preliminary studies had shown all samples to have very low concentrations of this proteinase.

4.3.4. Determination of BALF Histamine Concentration

BALF histamine concentrations were determined using a commercial competitive histamine ELISA (Immunotech) based on competition between histamine and its enzyme conjugate, histamine-alkaline phosphatase, for monoclonal anti-histamine antibody. To compete with this enzyme conjugate, histamine in the sample must be derivatised in the same manner as the histamine in the conjugate prior to analysis and this was achieved by an acylation step.

The kit was equilibrated to room temperature prior to use and all steps were performed using gloves to prevent contamination with extraneous histamine. All samples and standards were assayed in duplicate. The manufacturers state an intra-assay coefficient of variation of 7-10%, an inter-assay coefficient of variation of 8-13% and sensitivity down to 0.05ng/ml histamine.

Acylation was performed by thorough mixing of 25µl acylation buffer, 25µl acylation reagent and 100µl standards, control or samples. Acylated samples (50µl) were applied to the antibody coated microwells followed by 200µl enzymatic conjugate. Plates were incubated for 2h at 4°C, 350rpm (Stewart Scientific Bibby, Stewart Scientific) and then washed with 0.9% saline, 0.05% Tween 20 three times and dried by vigorously tapping the plate onto absorbent paper. Substrate solution (para-nitrophenylphosphate in diethanolamine-HCl solution, pH 9.8, 200µl) was added to wells and the plate incubated for 30min, 350rpm, room temperature. The reaction was then stopped by addition of 1N NaOH and the optical density of wells read by microplate reader at 405nm. Sample histamine concentrations were then interpolated from the standard curve.

4.3.5. Measurement of Tryptase Activity

As the ELISA will measure both active (tetrameric) and inactive (monomeric) tryptase, concentrations determined by ELISA give no measure of the proteolytic activity of BALF. Therefore BALF supernatant samples used for proteinase quantitation were also subjected to chromogenic assay for tryptase activity. Eq.MCP-

1 activity was not assessed, as preliminary trials showed that BALF samples contained very low concentrations of eq.MCP-1.

Samples were assayed at room temperature in ELISA plates using 50µl BALF sample, 45µl activity buffer (1M glycerol, 0.1M NaCl, 0.1M Tris-HCl and 100µg/ml heparin, pH 8.0) and 5µl chromogenic substrate S2366 (2.5mM). The substrate S2366 has previously been reported to be the most sensitive known substrate for equine tryptase (Pemberton *et al.*, 2001). A standard curve of tryptase concentrations typical of that found in equine BALF (5-60ng/ml) using activity buffer as diluent was assayed alongside samples to ensure that tryptase activity could be measured at these physiological concentrations. PBS was used as a negative control. Sample absorbance at 405nm was read by a microplate reader at t=0, 5, 10, 15, 20min and 24h. The increase in absorbance by 20min was minimal and therefore results were calculated from the 24h absorbance. Each sample was used as its own blank by subtracting absorbance at t=0 from final absorbance read at 24h and the rate calculated by the following equation:

$$\text{Activity rate (absorbance units / h)} = \frac{\text{absorbance t=24h} - \text{absorbance t=0}}{24}$$

4.3.6. Western Blotting of BALF Samples

4.3.6.1. Western Blots for Probing of Mast Cell Proteinases

Equine BALF supernatant (10µl), concentrated as previously described (4.3.2.2), or cell pellets with or without the addition of 0.1µg tryptase or eq.MCP-1 were heated with 11µl of reducing buffer for 5min. Samples were run on two SDS separating gels as described in section 2.3.1.3. Tryptase and eq.MCP-1 (0.1 µg) were also run as positive controls. One gel was stained with Coomassie blue for 15min and then destained as described in section 2.3.1.3 until bands could be visualised. The other gel was used for immunoblotting as described in section 2.4.1.

4.3.6.2. Western Blot to Determine Limit of Sensitivity of Mast Cell Proteinase Detection

In order to determine the limit of sensitivity of mast cell proteinase detection by Western blotting, a BALF cell pellet sample shown by ELISA to have a tryptase concentration of >200ng/ml was spiked with equine tryptase (0-1000ng) and subjected to SDS-PAGE. Western blotting and probing with 1μg/ml rabbit anti-equine tryptase were then performed as previously described.

4.3.7. Subjects and Collection of BALF for Immunolabelling

BALF samples (n=19) were collected as described previously (4.3.1.1) from controls (n=8), heaves horses (n=5) and horses with other pulmonary diseases (n=6) which had been referred to R(D)SVS for investigation of respiratory disease. Eight cytospins were made per BALF sample using a Shandon Cytospin 3 (100μl BALF, 300g, 3 min). Two cytospins were stained with Leishmans stain for DCC and the remaining slides fixed by immersion in acetone for 10min for later immunolabelling. Preliminary investigations with acetone, 4% paraformaldehyde and Carnoy's fixative determined that acetone was the most suitable fixative (data not shown).

4.3.8. Immunolabelling of BALF Cytospins

4.3.8.1. Trial Immunohistochemical Staining

Initial attempts at immunolabelling consisted of immunohistochemical staining with rabbit anti-equine tryptase / anti-eq.MCP-1 and normal rabbit IgG as a negative control. Briefly, slides were rehydrated in PBS, immersed in 97% methanol, 3% hydrogen peroxide to quench endogenous peroxidase activity, and then placed in Sequenza slide holders (Shandon). Non-specific binding was blocked with PBS, 0.5M NaCl, 0.5% Tween 80 for 30min. All further reagents were made up in this blocking buffer. Primary antibodies rabbit anti-equine tryptase / anti-eq.MCP-1 / normal rabbit IgG were applied at 1μg/ml for 1h. Slides were washed with PBS and the secondary antibody, biotinylated goat anti-rabbit IgG (Vector Laboratories) applied at 1/400 dilution for 30min. Following washing, avidin-horseradish

peroxidase conjugate (ABC reagent, Vector Laboratories) was added for 30min, then slides were washed and colour developed with 3,3'-diaminobenzidine (DAB kit, Vector Laboratories) for 2min. Slides were then washed, immersed in Mayer's haematoxylin (Sigma) for 5min, Scott's tap water for 2 min and then dehydrated through graded alcohols and xylene.

As staining was poor with this protocol, mouse monoclonal anti-human tryptase-biotin (clone AA5) and antigen retrieval by incubation in 0.1mg/ml trypsin in PBS at 37°C for 30min were also investigated, without success. Immunofluorescent labelling was therefore attempted.

4.3.8.2. Initial Immunofluorescent Labelling Protocol

Initially, cytospins were rehydrated in PBS and incubated with blocking buffer (PBS, 0.5M NaCl, 0.5% Tween 80, 10% normal donkey serum [Sigma]), for 1h to prevent non-specific binding. Rabbit anti-equine tryptase / anti-eq.MCP-1 / normal rabbit IgG were applied, 1μg/ml, for 1h and then slides washed and donkey anti-rabbit IgG conjugated to the green fluorochrome Alexafluor 488 (Invitrogen) was applied, at 4μg/ml, for 30min in the dark. Slides were washed with PBS and then coverslips mounted with Mowiol.

4.3.8.3. Final Tryptase Immunofluorescent Labelling Protocol

As some positive labelling of neutrophils and macrophages was observed with anti-equine tryptase labelling, the following dual immunofluorescence protocol was adopted for tryptase labelling.

After rehydration and blocking of slides as above, primary antibodies were applied overnight at 4°C as follows: 1μg/ml rabbit anti-equine tryptase + 2μg/ml sheep anti-equine neutrophil elastase-Cy3 (red fluorochrome; kindly donated by M. Dagleish) / 1μg/ml rabbit anti-equine tryptase + 2μg/ml normal sheep IgG-Cy3 (kindly donated by M. Dagleish) / 1μg/ml normal rabbit IgG + 2μg/ml sheep anti-equine neutrophil

elastase-Cy3. Slides were washed with PBS and then donkey anti-rabbit IgG-Alexafluor 488 applied as previously described (4.3.8.2).

4.3.8.4. Counting Protocol for Dual Immunofluorescence Cytospins

The number of tryptase or eq.MCP-1 positive mast cells per 500 (total) cells was counted under 488nm excitation at x400 magnification (Leica Laborlux S conventional compound microscope, Leica Microsystems). When positive cells were observed with anti-equine tryptase, cells were also viewed under 568nm excitation to assess anti-equine neutrophil elastase labelling, thus allowing differentiation between mast cells and neutrophils / macrophages.

4.3.8.5. Western Blot to Investigate Possible Cross Reactivity with Rabbit Anti-Equine Tryptase

Equine tryptase, elastase, trypsin and eq.MCP-1 (1µg / lane) were subject to SDS-polyacrylamide gel electrophoresis, western blotting and probing with 1µg/ml rabbit anti-equine tryptase as described in section 2.3.1.3 and 2.4.1.

4.3.8.6. Isolation and Immunofluorescent Labelling of Peripheral Blood Neutrophils

In order to further investigate the positive tryptase labelling of BALF neutrophils and macrophages, peripheral blood neutrophils were isolated and subject to dual immunofluorescent labelling. Neutrophils were isolated from peripheral blood using a discontinuous Percoll (Amersham Bioscience) gradient and centrifugation which separates cells according to their density. Briefly, blood was collected immediately post mortem from a horse euthanased for orthopaedic disease and citrated by addition of 1ml 3.8% sodium citrate / 10ml blood. Erythrocytes were allowed to sediment freely under gravity for 30min at room temperature at which point leukocyte rich plasma was removed and centrifuged at 380g for 6min. Pelleted cells were resuspended in 2ml platelet poor plasma (PPP) (previously made by centrifugation of plasma at 240g for 20min) and underlain by 2ml of 42% Percoll gradient (42% of 90% Percoll [90% Percoll, 10% sterile 0.9% saline] / 58% PPP)

followed by 2ml of 52% Percoll gradient (52% of 90% Percoll / 48% PPP). The resultant discontinuous gradient was centrifuged at 265g for 12min at room temperature allowing neutrophils to be harvested from the 52% / 42% interface. Neutrophils were washed in PPP and used to make cytospins (300g, 3min) which showed 98% purity of isolation. Neutrophils were washed twice in Hanks buffered salt solution without calcium and magnesium (Sigma), once in Hanks buffered salt solution with calcium and magnesium (Sigma) and were finally resuspended in the latter solution. Cytospins of purified peripheral blood neutrophils were prepared and immunolabelled as described in section 4.3.8.3.

4.3.8.7. Western Blot of Peripheral Blood Neutrophils

Isolated peripheral blood neutrophils were also subjected to SDS-polyacrylamide gel electrophoresis ($2x10^5$ cells / lane), western blotting and probing with rabbit anti-equine tryptase as described in sections 2.3.1.3. and 2.4.1. Equine neutrophil elastase was used as a positive control probe. Equine tryptase (1μg / lane) was also blotted and used as a positive control for the rabbit anti-equine tryptase probe.

4.3.9. Statistical Analyses

Statistical analyses were performed using Minitab 14 (Minitab). Normality of data was assessed using Anderson-Darling normality tests. Significance was taken at $p<0.05$ throughout. Outliers were defined as values differing from the middle 50% of data by a factor of 3 or more in either direction (Minitab).

4.3.9.1. ELISA CV

ELISA intra- and inter-assay CV were determined by expressing the standard deviation as a percentage of the mean for each group of values.

4.3.9.2. Data Transformation and Statistical Tests

All other data were analysed, where possible, using t-tests or analysis of variance (ANOVA) to allow possible confounding factors to be taken into account (section 4.3.9.3). The assumptions of ANOVA are that; (i) the data represents random sampling, (ii) data points are independent, (iii) groups have equal variance and (iv) the residuals follow a normal distribution (Crawley, 2002). If these assumptions were not met, attempts were made to normalize the data using square root or log transformation as appropriate (Crawley, 2002). If transformation did not result in the data meeting the assumptions for ANOVA, non-parametric statistics were performed using Kruskal Wallis tests for inter-group comparisons and Mann Whitney tests for two unpaired group comparisons. Only BALF absolute MC counts were normally distributed and therefore all other data were transformed as described. BALF tryptase concentration and activity rates were square root transformed. BALF absolute neutrophil count and immunolabelled BALF neutrophil data were log transformed. BALF eq.MCP-1 and histamine concentrations, and immunolabelled BALF MC data could not be transformed and were therefore subjected to non-parametric statistical analyses.

The relationship between two non-parametric data variables was examined by calculating the Spearman Rank Correlation Coefficient whereas the Pearson Product Moment Correlation Coefficient was calculated for parametric data variables. Regression analysis was performed to examine the relationship between two variables when the y variable depended upon the x variable and y was normally distributed.

4.3.9.3. Confounding Factors

As BALF proteinase and histamine data were drawn from 3 different populations, a number of confounding factors were likely to be present such as place, duration and type of challenge (natural vs. experimental) etc. These factors were examined using sequential univariate ANOVA models to examine their effect on the data. However, as some factors confounded each other (e.g. place of challenge and duration of

challenge), it was necessary to concentrate on the most important potential confounders which were deemed to be place of challenge, natural vs. experimental exposure and sampling time post challenge. Variables of interest were added into sequential multivariate ANOVA to examine data after taking significant confounders into account.

4.4. Results

4.4.1. Development of Mast Cell Proteinase ELISAs

4.4.1.1. Tryptase ELISA

Both mouse monoclonal anti-human tryptase-biotin (clone AA5) and polyclonal rabbit anti-equine tryptase successfully recognised equine tryptase. The mouse monoclonal antibody developed strong colour reaction at 1/1000 dilution but only a weak reaction at 1/5000 dilution. Polyclonal rabbit anti-equine tryptase developed a strong colour reaction at both 1 and 0.2μg/ml. However, no colour developed in the sandwich ELISA using polyclonal rabbit anti-equine tryptase as the coating antibody and mouse monoclonal anti-human tryptase-biotin (clone AA5) as the secondary antibody after incubation for 1h at room temperature or overnight at 38°C, indicating possible competition of antibodies for the same epitope.

Mouse monoclonal anti-human tryptase (clone AA1) appeared to have no specificity for equine tryptase. The sandwich ELISA using this monoclonal antibody as the coating antibody and polyclonal rabbit anti-equine tryptase as the secondary antibody showed only a weak colour change with 1/100, 1/500 and 1/1000 dilutions of primary antibody.

Biotinylated rabbit polyclonal anti-equine tryptase showed optimal colour development at 1μg/ml with a linear curve up to 10ng/ml tryptase with a mean and standard deviation standard curve correlation coefficient of 0.97 ± 0.01 (n=23).

4.4.1.2. Eq.MCP-1 ELISA

Rabbit polyclonal anti-eq.MCP-1 and biotinylated rabbit polyclonal anti-eq.MCP-1 both reacted positively with eq.MCP-1 coated wells. The sandwich ELISA using these antibodies showed linear development of colour along the standard curve up to 20ng/ml and a mean and standard deviation standard curve correlation coefficient of 0.99 ± 0.01 (n=5).

Unconcentrated BALF samples appeared to have very low eq.MCP-1 concentrations as eq.MCP-1 was undetectable in 5 samples analysed by ELISA. There was no background colour to the eq.MCP-1 ELISA.

4.4.2. Validation of Proteinase ELISAs

4.4.2.1. Control ELISA Experiment

Very little non-specific background colour was attributable to reagents used in either the tryptase or the eq.MCP-1 ELISAs (table 4.3).

Sample	Tryptase OD	Eq.MCP-1 OD
Positive control	0.177	0.363
No coating antibody	0.042	0.043
No tryptase	0.041	0.040
No secondary antibody	0.035	0.041
No S-HRP	0.039	0.040
No reagents	0.022	0.024

Table 4.3: Optical density (OD) of samples in control ELISA experiment. S-HRP streptavidin horse radish peroxidase.

4.4.2.2. Spiked BALF

The mean (range) spiked BALF tryptase concentration read by the ELISA was 83% (53-118%) of the calculated value for low spiked tryptase concentrations and 195% (74-362%) for high spiked concentrations. Actual spiked concentrations and ELISA

readings for the low tryptase spiked concentration are shown in table 4.4 and high tryptase spiked concentrations are shown in table 4.5.

Unconcentrated Apparent BALF [tryptase] (ng/ml)	Tryptase spike (ng/ml)	Spiked BALF [tryptase] (ng/ml)	ELISA BALF [tryptase] (ng/ml)	% Actual Value
4.9	5	9.9	8.8	89
1.7	10	11.7	6.2	53
1.7	8	9.7	5.5	57
1.7	6	7.7	5.5	71
1.7	4	5.7	4.4	77
1.7	2	3.7	2.9	78
1.7	1	2.7	2.5	93
1.7	0.5	2.2	2.5	114
1.7	0.25	1.95	2.3	118
Mean				**83**

Table 4.4: Calculated concentrations and ELISA readings of BALF spiked with low concentrations of tryptase.

[Spike] (ng/ml)	Dilution Factor	[Tryptase] (ng/ml)	% Actual Value
1000	0	> scale	N/A
	10	> scale	N/A
	100	942.1	94
	1000	2184.0	218
500	0	> scale	N/A
	10	940.1	188
	100	761.7	152
	1000	2106	421
100	0	> scale	N/A
	10	74.2	74
	100	149.8	150
	1000	< scale	N/A
50	0	> scale	N/A
	10	47.1	94
	20	78.2	156
	100	181.1	362
25	0	> scale	N/A
	10	31.8	127
	20	37.4	150
	100	87.0	348
Mean			**195**

Table 4.5: ELISA readings of high concentration tryptase spiked BALF. N/A = not applicable, > scale = ELISA reading above the top of the standard curve, < scale = ELISA reading below the bottom of the standard curve.

The mean (range) spiked BALF eq.MCP-1 concentration read by the ELISA was 184.4% (93.3-431.8) of the spiked concentration as shown in table 4.6. As unconcentrated BALF samples appeared to contain very little eq.MCP-1, further manipulation of this ELISA was not performed.

[Spike] (ng/ml)	Dilution Factor	[Eq.MCP-1] (ng/ml)	% Actual Value
1000	0	> scale	N/A
	10	> scale	N/A
	100	1399.4	140
	1000	1609.7	161
500	0	> scale	N/A
	10	> scale	N/A
	100	892.2	178
	1000	2159.2	432
100	0	> scale	N/A
	10	101.9	102
	100	93.3	93
	1000	< scale	N/A
Mean			**184**

Table 4.6: ELISA readings of eq.MCP-1 spiked BALF. N/A = not applicable, > scale = ELISA reading above the top of the standard curve, < scale = ELISA reading below the bottom of the standard curve.

4.4.2.3. Background Colouration in Tryptase ELISA

Blocking with ELISA buffer prior to addition of the secondary antibody did not improve ELISA determination of tryptase concentrations of unconcentrated BALF serial dilutions (table 4.7).

	Sample 1		*Sample 2*	
	no blocking	***blocking***	***no blocking***	***blocking***
Dilution Factor	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*
0	2.3	3.1	1.7	1.6
1.3	2.4	2.8	2.3	2.6
2.0	3.1	5.4	3.2	3.6
4.0	4.8	5.7	5.6	8.2
6.7	N/A	N/A	6.2	6.7
10.0	N/A	N/A	10.0	9.0
20.0	N/A	N/A	12.0	8.0
100.0	N/A	N/A	< scale	16.8

Table 4.7: ELISA determined tryptase concentrations of serial dilutions of unconcentrated BALF with and without a blocking step of ELISA buffer prior to addition of secondary antibody. N/A = not applicable, < scale = ELISA reading below the bottom of the standard curve.

Addition of 0.5mg/ml DTT also did not reduce the background colouration obtained in the ELISA using unconcentrated BALF from control or heaves affected horses (table 4.8). At higher, spiked tryptase concentrations, DTT appeared to adversely affect the ELISA causing underestimation of tryptase readings (table 4.9).

	Unconcentrated Control BALF		***Unconcentrated Heaves BALF***	
	No DTT	***0.5mg/ml DTT***	***No DTT***	***0.5mg/ml DTT***
Dilution Factor	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*
0	3.3	2.9	3.4	2.8
1.3	3.5	2.8	3.7	2.9
2	4.9	4.7	5.4	4.0
4	6.2	7.1	7.0	6.3
10	9.9	11.4	10.6	9.3
20	10.4	10.8	13.3	6.7
50	28.0	17.1	29.9	< scale
100	< scale	32.4	< scale	< scale

Table 4.8: ELISA determined tryptase concentrations in raw BALF from control and heaves affected horses with and without treatment with 0.5mg/ml dithiothreitol (DTT). < scale = ELISA reading below the bottom of the standard curve.

		Spiked Control BALF		*Spiked Heaves BALF*	
		No DTT	***0.5mg/ml DTT***	***No DTT***	***0.5mg/ml DTT***
[Spike] (ng/ml)	*Dilution Factor*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*
100	0	> scale	9.9	> scale	6.9
	10	92.9	19.6	> scale	21.4
	100	141.0	34.2	116.9	40.5
	1000	< scale	< scale	< scale	< scale
50	0	> scale	7.8	> scale	> scale
	10	51.2	11.0	63.2	12.0
	20	44.4	14.8	59.4	13.7
	100	61.1	36.0	98.6	61.3

Table 4.9: ELISA determined tryptase concentrations in spiked BALF from control and heaves affected horses with and without treatment with 0.5mg/ml dithiothreitol (DTT). > scale = ELISA reading above the top of the standard curve, < scale = ELISA reading below the bottom of the standard curve.

High speed centrifugation at 20,000g for 10min and filtration of BALF to remove mucus strands also did not produce consistent improvement in ELISA tryptase readings (table 4.10).

	Control BALF			*Heaves BALF*		
	N	***C***	***C & F***	***N***	***C***	***C & F***
Dilution Factor	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*	*[tryptase] (ng/ml)*
0	9.9	7.6	7.8	> scale	> scale	> scale
10	14.2	8.9	4.6	> scale	> scale	> scale
50	< scale	< scale	< scale	384.6	533.5	304.8
100	< scale	< scale	< scale	454.4	652.4	431.4

Table 4.10: ELISA determined tryptase concentrations in control and heaves BALF with no treatment (N), centrifugation at 20,000g, 10min (C), or centrifugation at 20,000g, 10min plus filtration through a 0.45µm filter (C & F). > scale = ELISA reading above the top of the standard curve, < scale = ELISA reading below the bottom of the standard curve.

Performance of the tryptase ELISA was not consistently improved by any of the methods investigated. ELISA determination of tryptase concentrations was most consistent with BALF of lower proteinase concentrations and when minimal dilutions were used for higher tryptase concentrations. Therefore, when dilution of samples was necessary, the minimal dilution to achieve a reading within the range of the standard curve was used.

4.4.2.4. Heparin-Agarose Tryptase ELISA

Incubation with a heparin-agarose saline slurry reduced the tryptase concentration by 68% in the BALF sample and by 44% in the tryptase-spiked saline sample (table 4.11). The negative control saline diluted BALF (diluted with saline equivalent to that added with heparin-agarose slurry) showed a tryptase reduction of 25%, very close to the predicted 33% expected from the dilution factor. In this case, the positive control tryptase-spiked saline contained 66.7ng/ml and therefore the ELISA slightly underestimated the tryptase concentration (51.7ng/ml).

	BALF		***Spiked Saline (66.7ng/ml)***	
Treatment	*[tryptase] (ng/ml)*	*Reduction in tryptase (%)*	*[tryptase] (ng/ml)*	*Reduction in tryptase (%)*
-	16.0	N/A	51.7	N/A
Heparin Agarose	5.1	68	28.8	44
Saline dilution	11.7	27	N/A	N/A

Table 4.11: ELISA determined tryptase concentrations of BALF and tryptase-spiked saline with and without addition of heparin-agarose to remove tryptase from solution. N/A = not applicable.

4.4.2.5. Coefficients of Variation

Intra-plate and inter-plate CVs for the tryptase ELISA were 20.4% and 22.1% for control horse samples and 10.8% and 24.1% for heaves horse samples, respectively. Intra-plate and inter-plate CV for the eq.MCP-1 ELISA were 14.9% and 23.6%, respectively.

4.4.3. BALF Tryptase Concentrations

The ELISA method described above was used to compare tryptase concentrations in BALF from control, heaves, remission and other disease horses. Following square root transformation, the residuals of the tryptase data set assumed a normal distribution apart from 4 samples (3 from the heaves group and 1 from the other disease group) with very high tryptase values (>70ng/ml). Log transformation did not result in these residuals assuming a normal distribution. The order of mean tryptase values for control, remission, other disease and heaves groups of horses were unaffected by omission of these 4 values (3.1, 5.1, 20.4, 22.5ng/ml and 3.1, 5.1, 7.1, 8.3ng/ml, respectively before and after exclusion). This is demonstrated by the graph of raw data tryptase concentrations (fig. 4.1) being qualitatively the same as the graph of normalised tryptase values (fig. 4.2). These four data points were therefore excluded from statistical analyses. Furthermore, despite exclusion of these values, one-way ANOVA of normalised BALF tryptase concentrations revealed a highly significant difference among groups ($p<0.001$) (fig. 4.2).

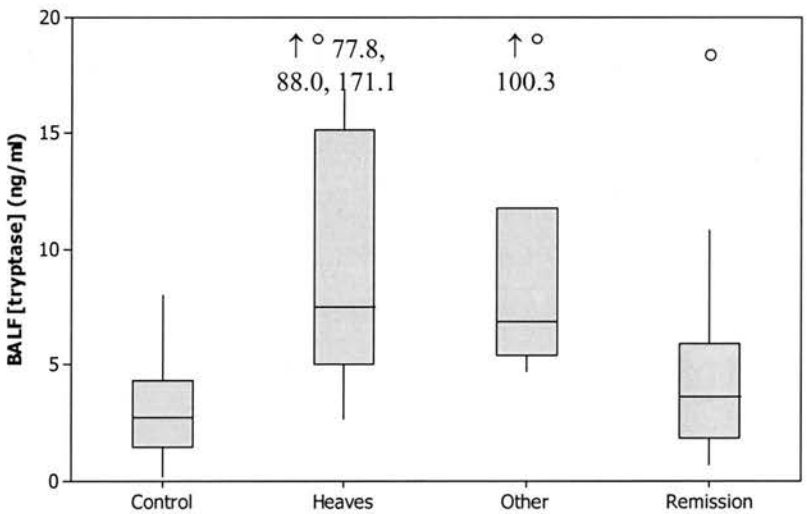

Fig. 4.1: BALF tryptase concentrations (ng/ml) in control (n=22), heaves (n=21), other disease (n=7) and remission (n=13) horses. ° denotes statistical outlier, ↑ ° denotes outlier off axis scale.

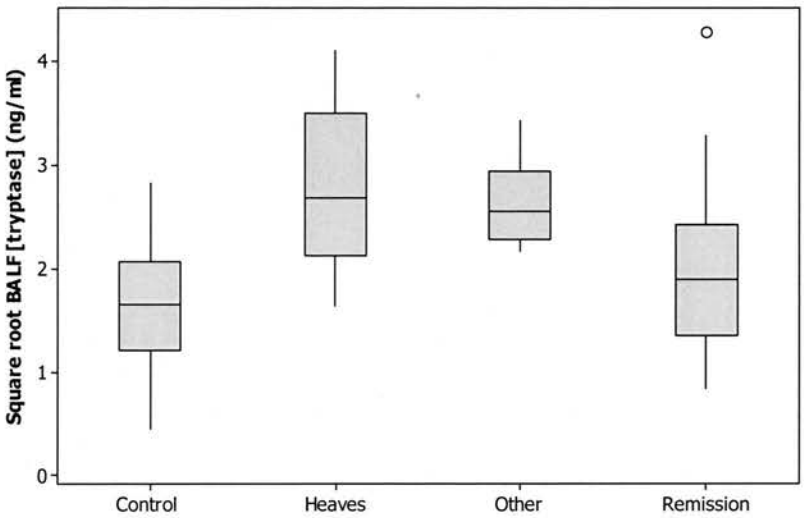

Fig. 4.2: Normalised (square root) BALF tryptase concentrations (ng/ml) in control (n=22), heaves (n=18), other disease (n=6) and remission (n=13) horses. ANOVA revealed a highly significant difference in BALF tryptase concentration among groups ($p<0.001$). ° denotes outlier.

Since the sample population was derived from several experiments, the effect of potential confounding factors (fig. 4.3) on the data was initially examined separately using univariate ANOVAs as discussed in section 4.3.9.3.

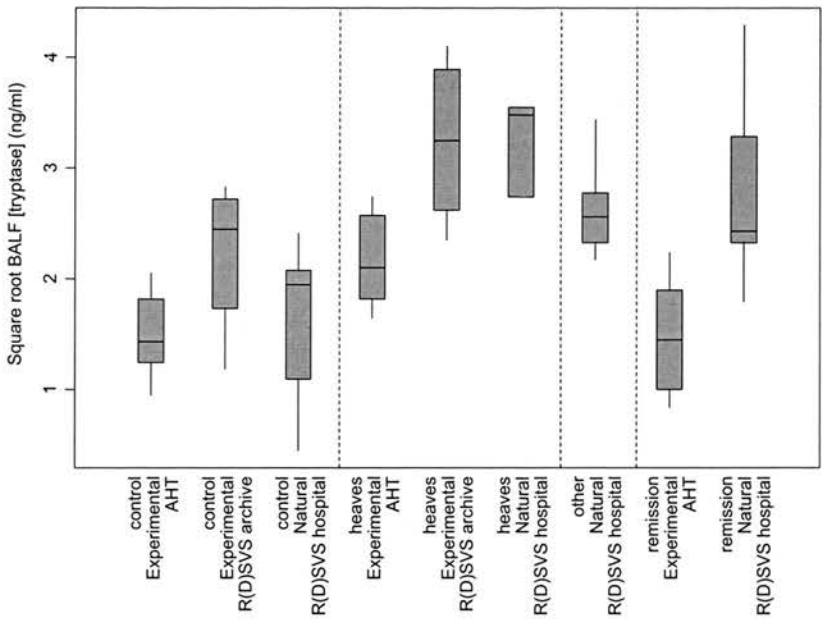

Fig. 4.3: Normalised BALF tryptase concentrations (ng/ml) of control (n=22), heaves (n=18), other disease (other) (n=6) and remission (n=13) horses showing confounding factors of place and type of challenge. Samples were obtained from three studies; AHT archive (AHT) (n=26), R(D)SVS archive (n=13) and R(D)SVS hospital (n=20). Horses received either natural (n=20) or experimental (n=39) challenge.

These univariate ANOVA revealed that BALF tryptase concentrations were significantly different with respect to time of sampling ($F_{2,58}$=14.7, p<0.001) (fig. 4.4) and place of challenge ($F_{2,58}$=15.2, p<0.001) (fig. 4.5). The three challenges differed in duration: R(D)SVS archive study, 5h; AHT archive study, 24h; R(D)SVS hospital, chronic challenge. Duration of challenge was found to have a highly significant effect on BALF tryptase concentrations ($F_{3,58}$=12.3, p<0.001) (fig. 4.6). Type of challenge (natural vs. experimental) did not cause a significant difference in tryptase concentration ($F_{1,58}$=3.4, p=0.07) (fig. 4.7).

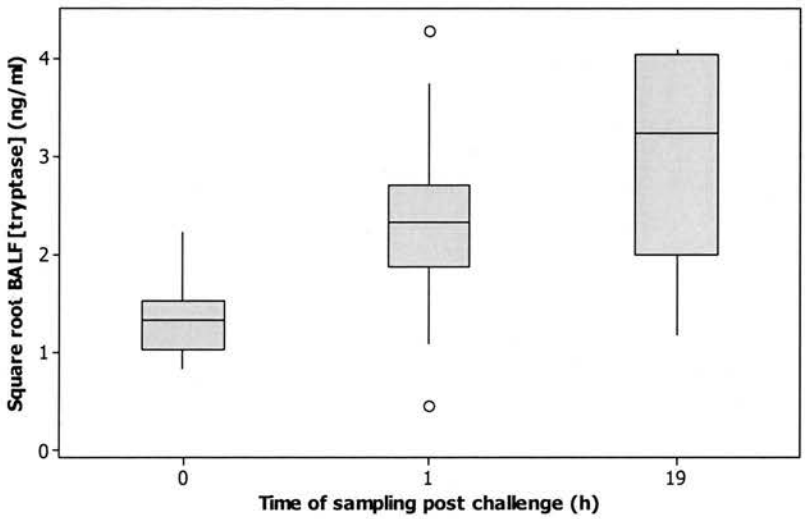

Fig. 4.4: Normalised BALF tryptase concentrations (ng/ml) of horses sampled immediately (0) (n=13), 1h (n=40) or 19h (n=6) following end of challenge. One-way ANOVA revealed a highly significant difference among groups ($p<0.001$). ° denotes outlier.

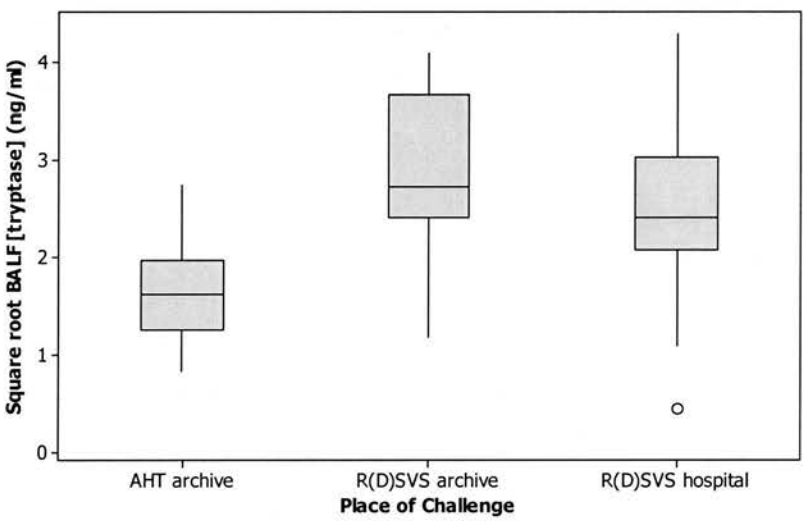

Fig. 4.5: Normalised BALF tryptase concentration (ng/ml) in AHT archive study (n=26), R(D)SVS archive study (n=13) and R(D)SVS hospital (n=20). One-way ANOVA revealed a highly significant difference among groups ($p<0.001$). ° denotes outlier.

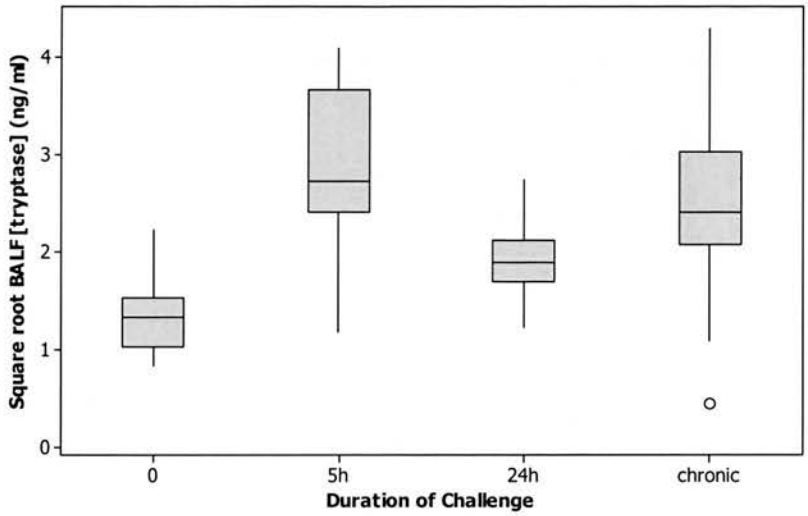

Fig. 4.6: Normalised BALF tryptase concentration (ng/ml) following no challenge (0) (n=13), 5h (n=13), 24h (n=13) or chronic (n=20) challenge. One-way ANOVA revealed a highly significant difference among groups ($p<0.001$). ° denotes outlier.

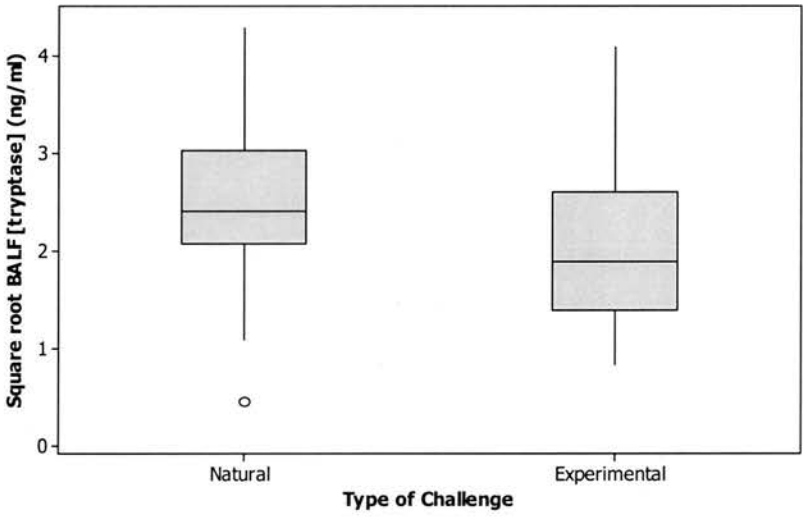

Fig. 4.7: Normalised BALF tryptase concentration (ng/ml) following natural (n=20) or experimental (n=39) hay / straw challenge. One-way ANOVA revealed no significant difference between groups ($p=0.07$). °

Sequential multivariate ANOVA taking these significant confounding factors into account showed that there was no significant difference in BALF tryptase concentrations between control and challenged control horses ($F_{1,18}$=2.5, p=0.13). Data for control and challenged control horses were therefore pooled. Additional multivariate ANOVA, again taking significant confounders into account, revealed there was a highly significant difference in BALF tryptase concentration among disease groups ($F_{3,52}$=8.1, p<0.001). Multivariate ANOVAs for specific group comparisons showed that despite confounding factors, there was still a significant difference in BALF tryptase concentrations between control and heaves horses ($F_{1,35}$=26.1, p<0.001), heaves and remission horses ($F_{1,26}$=5.4, p=0.03) and control vs. other disease horses ($F_{1,23}$=11.4, p=0.002) (fig. 4.8). There was no significant difference in tryptase concentrations between control and remission horses ($F_{1,30}$=3.2, p=0.08) or other disease and remission horses ($F_{1,16}$=0.1, p=0.74).

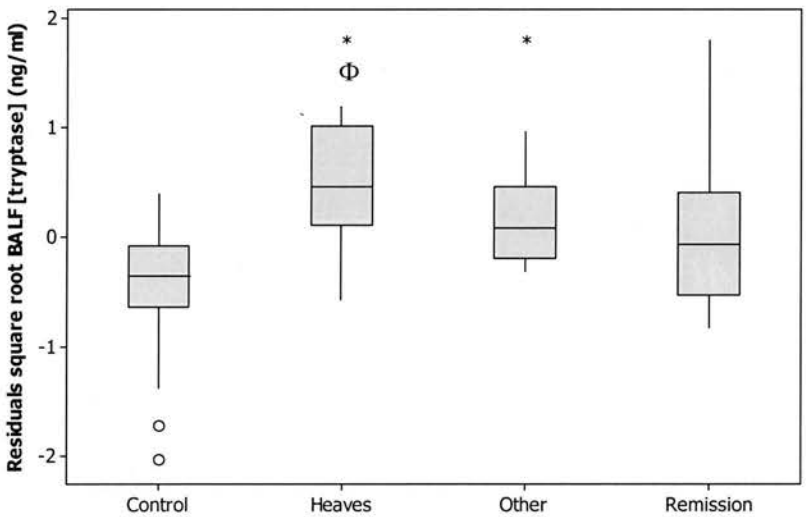

Fig 4.8: Normalised BALF tryptase concentration (ng/ml) of residuals in control (n=22), heaves (n=18), other disease (n=6) and remission (n=13) horses following removal of the effect of time of sampling post challenge and place of challenge. * denotes significant difference to control (p<0.01), Φ denotes significant difference to remission horses (p<0.05).

BALF absolute neutrophil count residuals assumed a normal distribution following log transformation. Univariate ANOVA revealed that there was no significant difference in the absolute BALF neutrophil count of heaves susceptible horses post

challenge among the three different places of challenges ($F_{2,18}$ = 0.27, p=0.76) (fig. 4.9) suggesting that all three challenges presented an equivalent provocative insult.

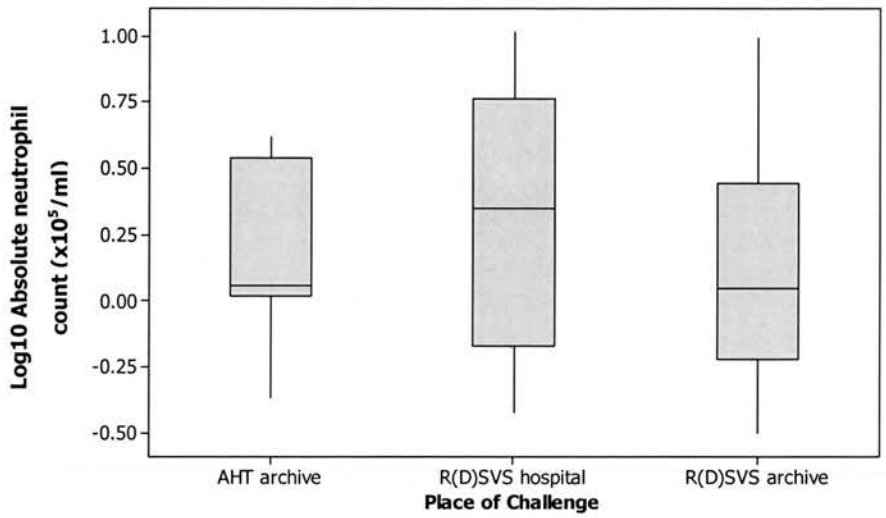

Fig: 4.9: Log10 post challenge BALF absolute neutrophil counts of heaves susceptible horses in AHT archive (n=7), R(D)SVS hospital (n=5) and R(D)SVS archive (n=9). There was no significant difference in neutrophil count among groups.

Univariate ANOVA also revealed that type of challenge (natural vs. experimental) and time of sampling post challenge had no significant effect on BALF absolute neutrophil response ($F_{1,19}$=0.49, p=0.49; $F_{1,19}$=2.09, p=0.16 respectively). Duration of challenge had no significant effect on post challenge BALF absolute neutrophil count ($F_{2,18}$ = 0.27, p=0.76). In contrast, a highly significant effect of duration of challenge on tryptase concentration in heaves susceptible horses ($F_{2,15}$=8.6, p=0.003) (fig. 4.10) was evident, but there was no apparent trend.

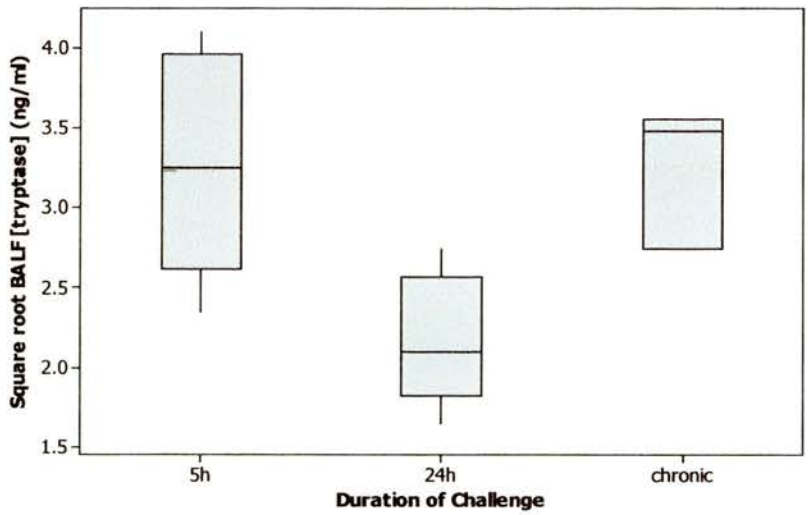

Fig: 4.10: Square root BALF tryptase concentration (ng/ml) in heaves susceptible horses post 5h (R[D]SVS archive) (n=8), 24h (AHT archive) (n=7) or chronic (R[D]SVS hospital) (n=3) challenge. There was a significant difference in tryptase concentration with duration of challenge (p=0.003).

Although individual group correlations were not significant, there was a highly significant correlation between BALF tryptase concentration and BALF absolute neutrophil count within the complete dataset (Pearson r=0.53, p<0.001, n=55, neutrophil counts missing for 4 horses) (fig. 4.11).

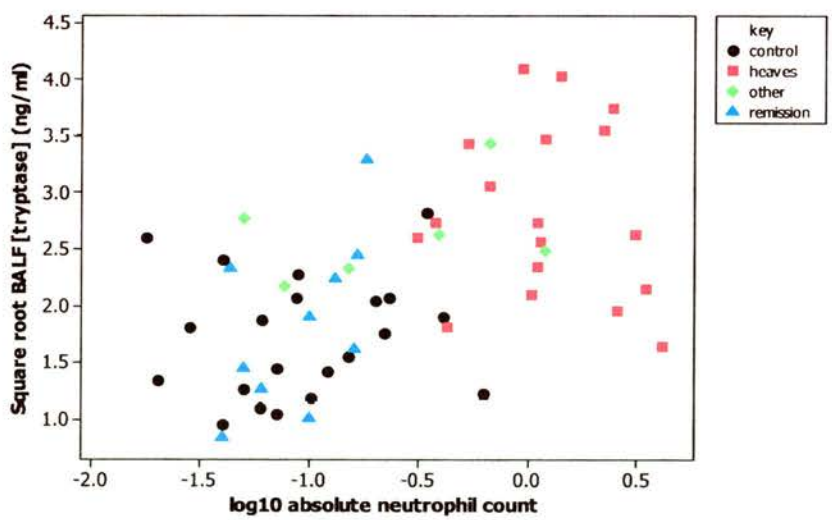

Fig. 4.11: Scatterplot showing positive correlation of BALF absolute neutrophil count ($x10^5$/ml) and BALF tryptase concentration (n=55), Pearson r=0.53, p<0.001.

Absolute mast cell counts were only available for 33 samples. There was no significant relationship between BALF tryptase concentration and absolute mast cell count (t=0.71, p=0.49, R^2=1.8%).

One set of archived material (AHT archive) consisting of baseline and post 24h challenge samples represented paired data. Paired t-tests revealed a significant increase in BALF tryptase concentration in both control (t=-6.55, p=0.001) (fig. 4.12) and heaves susceptible horses (t=-3.1, p=0.02) (fig. 4.13) following challenge.

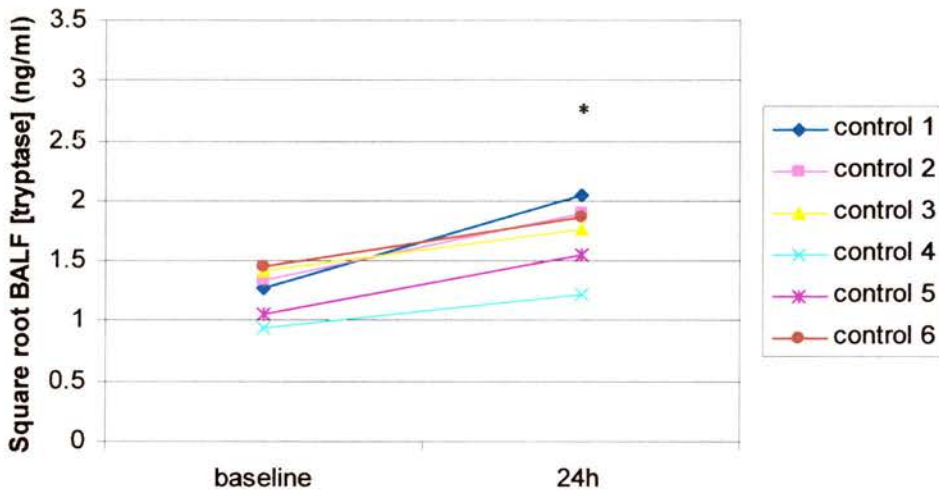

Fig. 4.12: Normalised BALF tryptase concentration in AHT archive control horses (n=6) at baseline and immediately post 24h challenge, * denotes significant difference between baseline and post challenge values (p=0.001).

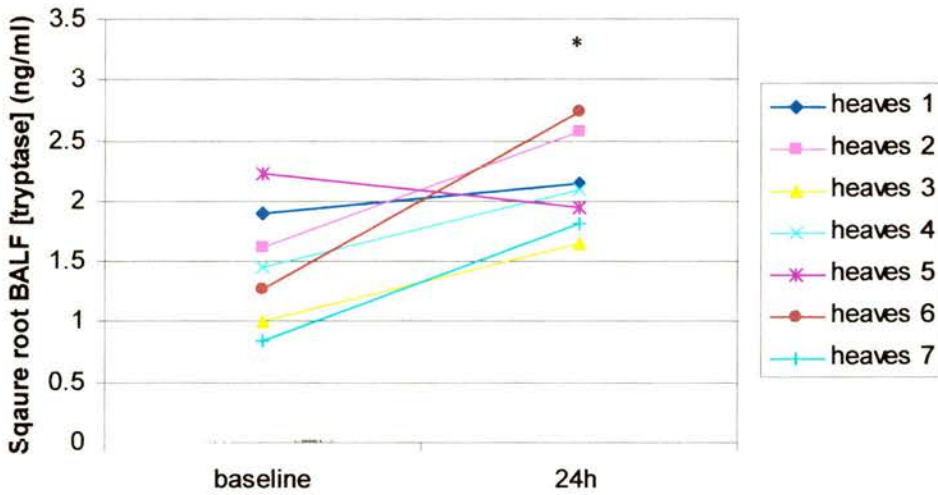

Fig. 4.13: Normalised BALF tryptase concentration in AHT archive heaves susceptible horses (n=7) at baseline and immediately post 24h challenge, * denotes significant difference between baseline and post challenge values (p=0.02).

Two sample t-tests revealed no significant difference in BALF tryptase concentration between this subset of control and heaves susceptible horses at baseline (t=-1.1, p=0.3) (fig. 4.14).

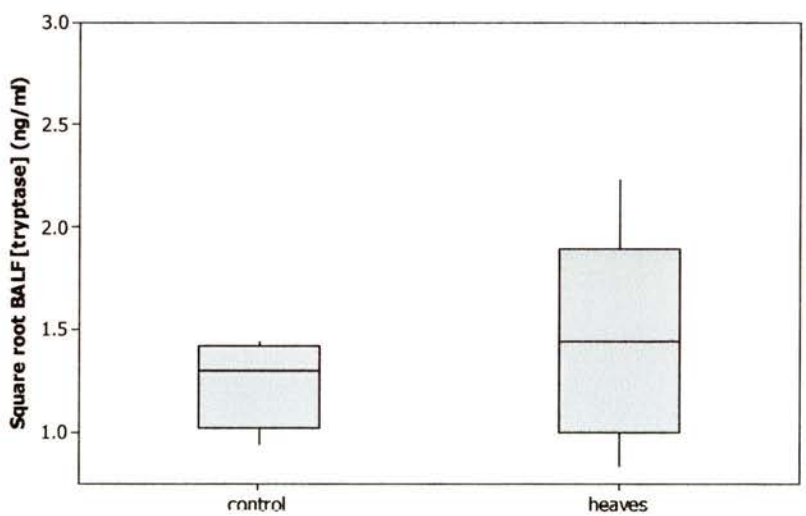

Fig. 4.14: Normalised BALF tryptase concentrations (ng/ml) of AHT archive control (n=6) and heaves susceptible (n=7) horses at baseline.

A strong trend was evident towards a significant difference between control and heaves horses post challenge (t=-2.2, p=0.057) (fig. 4.15), however, there was no significant difference between control and heaves horses in their response to challenge (t=-0.82, p=0.44).

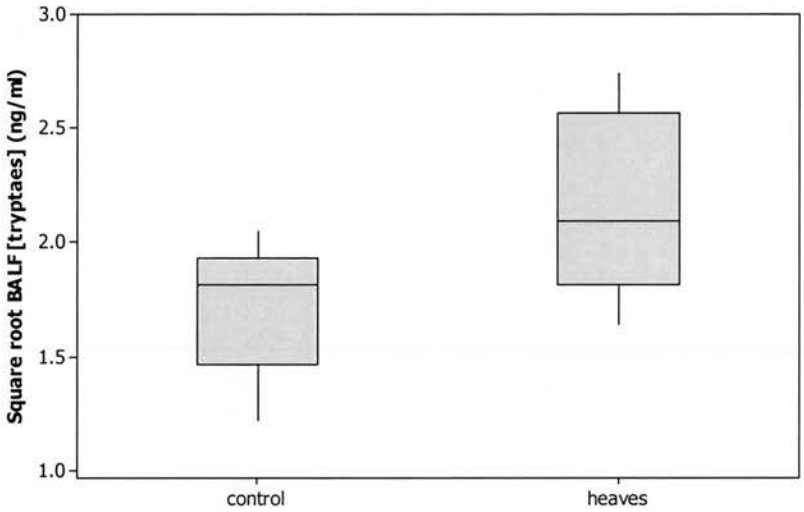

Fig. 4.15: Normalised BALF tryptase concentrations (ng/ml) of AHT archive control (n=6) and heaves susceptible (n=7) horses post 24h hay / straw challenge. There was no significant difference between control and heaves horses (p=0.057).

4.4.4. BALF Eq.MCP-1 Concentrations

Very low (<3ng/ml) (n=28, 44%) or undetectable (<0.25ng/ml) (n=35, 56%) concentrations of eq.MCP-1 were measured in BALF samples. Due to the high proportion of samples with undetectable eq.MCP-1, this data could not be successfully transformed and therefore Kruskal Wallis analysis was performed. There was no significant difference in eq.MCP-1 among groups of horses (p=0.47) (fig. 4.16) and therefore no further statistical analysis was performed.

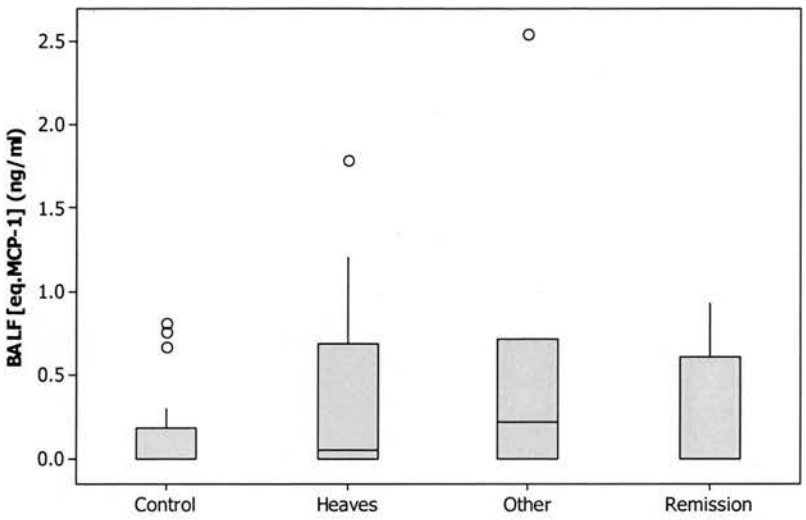

Fig. 4.16: BALF eq.MCP-1 concentration (ng/ml) in control (n=22), heaves (n=21), other disease (n=7) and remission (n=13) horses. Kruskal-Wallis analysis revealed no significant difference in eq.MCP-1 concentration among groups, ° denotes outlier.

4.4.5. BALF Histamine Concentrations

Histamine was undetectable (<0.05ng/ml) in 42 (67%) samples and ranged from 0.1-7.5ng/ml in the remaining 21 (33%) samples. Again, transformations were unsuccessful in making the residuals of this data set assume a normal distribution due to the majority of samples having undetectable values for BALF histamine concentration. Kruskal Wallis analysis was therefore performed and revealed no significant difference in histamine among groups of horses (p=0.26) (fig. 4.17).

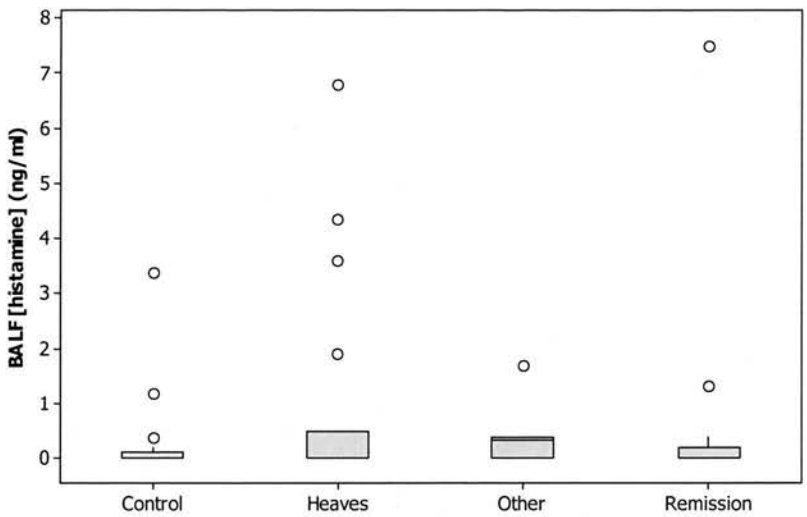

Fig. 4.17: BALF histamine concentration (ng/ml) in control (n=22), heaves (n=21), other disease (n=7) and remission (n=13) horses. There was no significant difference in histamine concentration among groups (p=0.26), ° denotes outlier.

As histamine is very labile and since BALF samples may have been processed differently at the three places of challenge, samples were divided into place of origin. Subsequent Kruskal Wallis analysis revealed that BALF histamine concentrations were significantly different among places of challenge (p<0.001) (fig. 4.18).

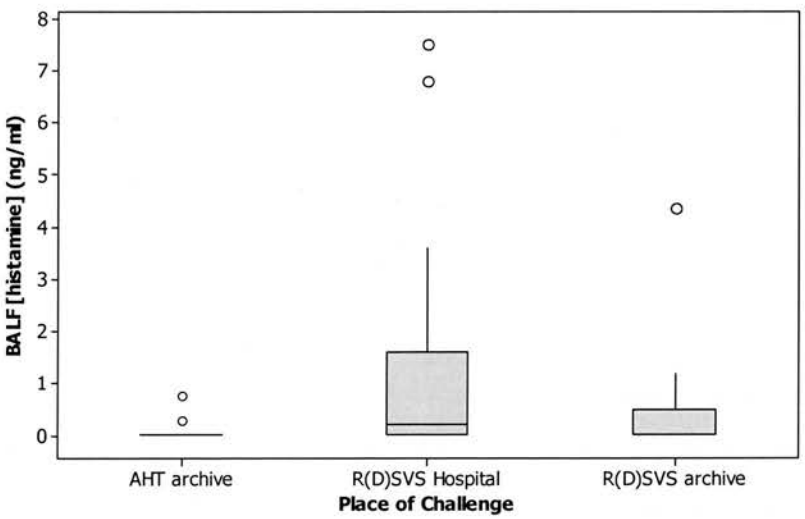

Fig. 4.18: Histamine concentration (ng/ml) of BALF samples from AHT archive (n=26), R(D)SVS archive (n=13) and R(D)SVS hospital (n=24) groups. Kruskal Wallis analysis revealed a highly significant difference among groups (p<0.001). ° denotes outlier.

Because variable degrees of histamine degradation prior to sample analysis could have accounted for some of the inter-group difference in histamine concentrations, data were divided into samples with minimal storage and only one freeze–thaw cycle (R[D]SVS hospital) and archived samples (AHT and R[D]SVS archived studies). These archived samples had been stored for >1yr and possibly subjected to multiple freeze-thaw episodes. Mann Whitney analysis showed that archived samples had significantly less histamine than hospital samples ($p<0.001$) (fig. 4.19).

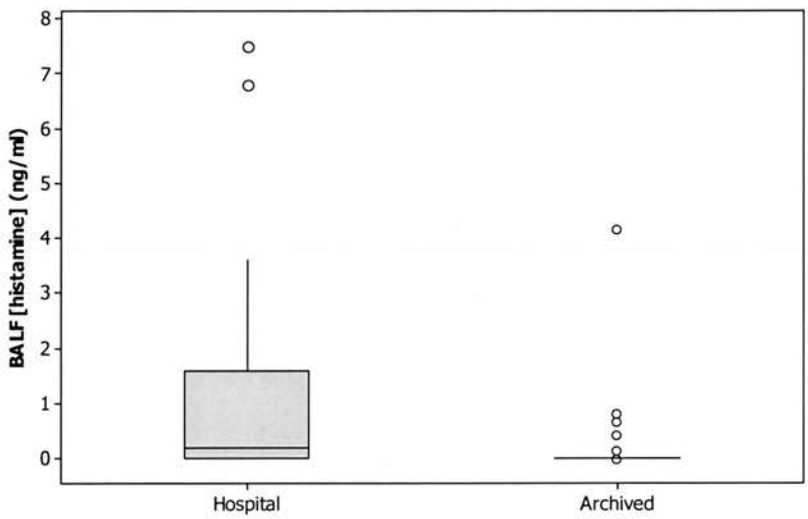

Fig. 4.19: Histamine concentration (ng/ml) of BALF samples from R(D)SVS hospital group (hospital, n=24) and from archived AHT and R(D)SVS studies (archived, n=39). Archived samples had significantly less histamine ($p<0.001$), ° denotes outlier.

Due to this apparent effect of storage on histamine concentration, the R(D)SVS hospital subset of data was analysed separately by Kruskal Wallis test. Due to the wide range in histamine concentrations, there was no significant difference in BALF histamine concentrations with disease status ($p=0.8$) (fig. 4.20).

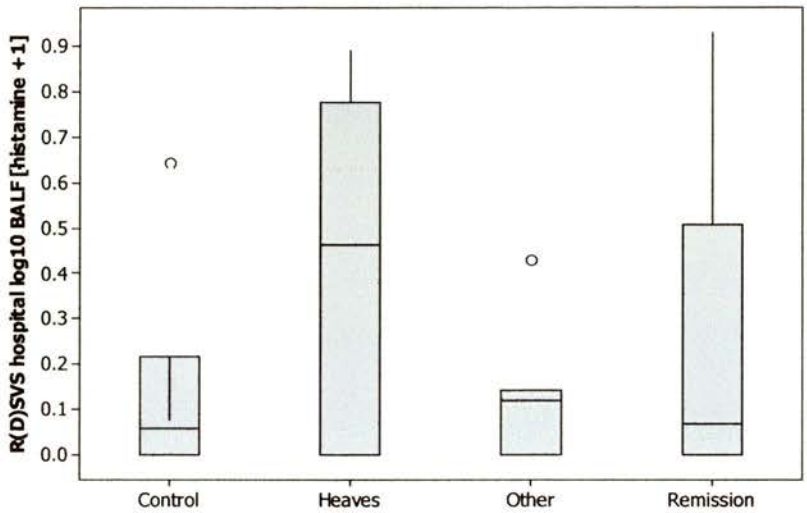

Fig. 4.20: BALF histamine concentrations (ng/ml) in the R(D)SVS hospital subset of control (n=6), heaves (n=5), other disease (n=7) and remission (n=6) horses. There was no significant difference in histamine concentration among groups (p=0.8).

Within this R(D)SVS hospital dataset, there was a suggestion of a trend for a correlation between BALF tryptase and histamine concentrations (Spearman r=0.36, p=0.10) (fig. 4.21).

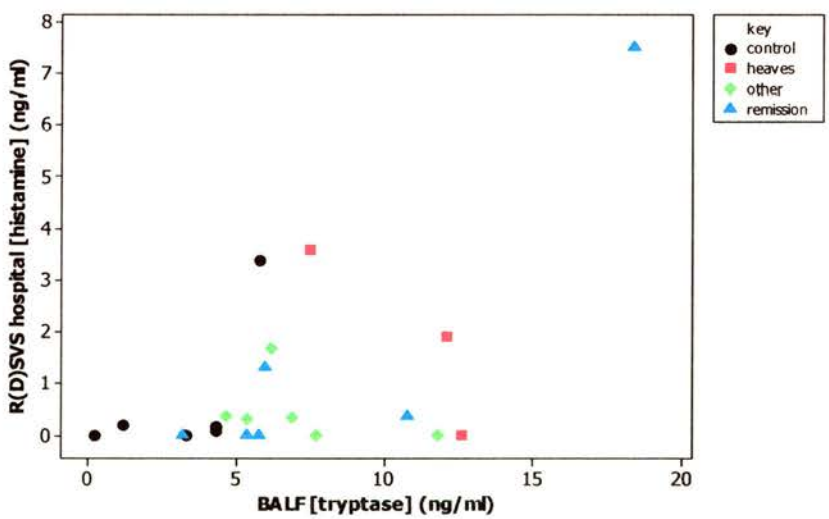

Fig. 4.21: Scatterplot showing weak positive correlation of BALF tryptase and histamine concentrations in the R(D)SVS hospital dataset (n=21), Spearman r=0.36, p=0.10.

Regression analysis to explore the relationship of BALF histamine with absolute mast cell count for this data subset could not be performed as the histamine data could not be normalised due to the high proportion of zero values.

4.4.6. Tryptase Activity

4.4.6.1. Standards

Tryptase activity rates were measurable in the standards representing physiological BALF concentrations. Regression analysis of these standards showed that activity rate was positively related to tryptase concentration ($r=0.89$, $p=0.046$) (fig. 4.22).

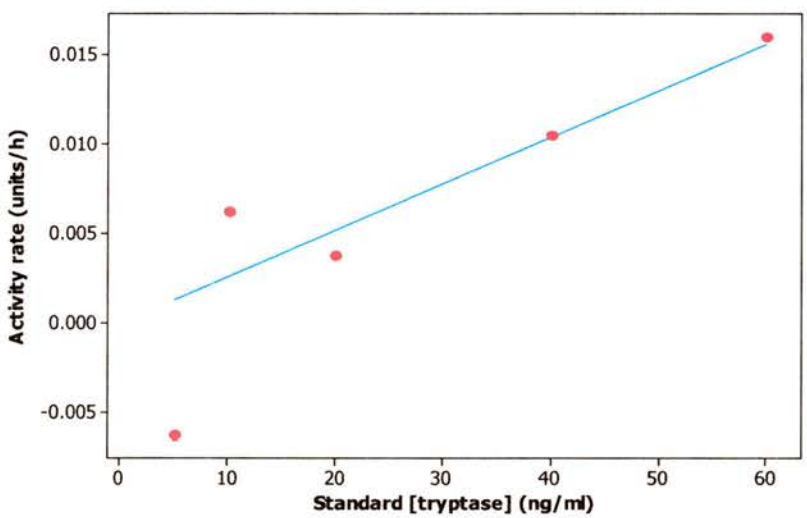

Fig. 4.22: Activity rate (units/h) of tryptase standards as a function of tryptase concentration.

4.4.6.2. BALF Samples

BALF tryptase activity residuals were normalised by square root transformation. This excluded data from 4 horses which had apparent negative BALF tryptase activity rates. Univariate ANOVA showed that there was a strong trend for a significant inter-group difference in BALF tryptase activity ($F_{3,51}=2.77$, $p=0.051$) (fig. 4.23).

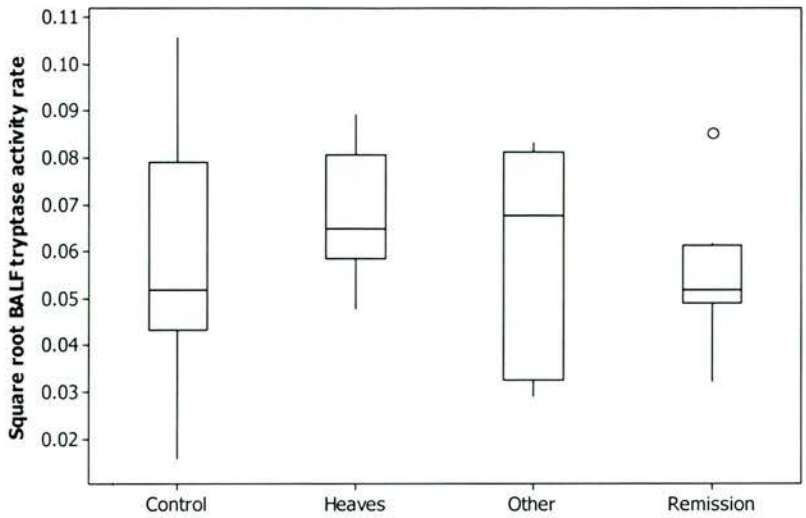

Fig. 4.23: BALF tryptase activity rates in control (n=20), heaves (n=17), other disease (n=7) and remission (n=11) horses. One-way ANOVA revealed a strong trend for an inter-group variation in BALF tryptase activity (p=0.051). ° denotes outlier.

Multivariate ANOVA allowing for the effects of time of sampling and place of challenge showed that BALF tryptase activity was significantly greater in heaves horses than remission horses ($F_{1,23}$=4.9, p=0.04) (fig. 4.24).

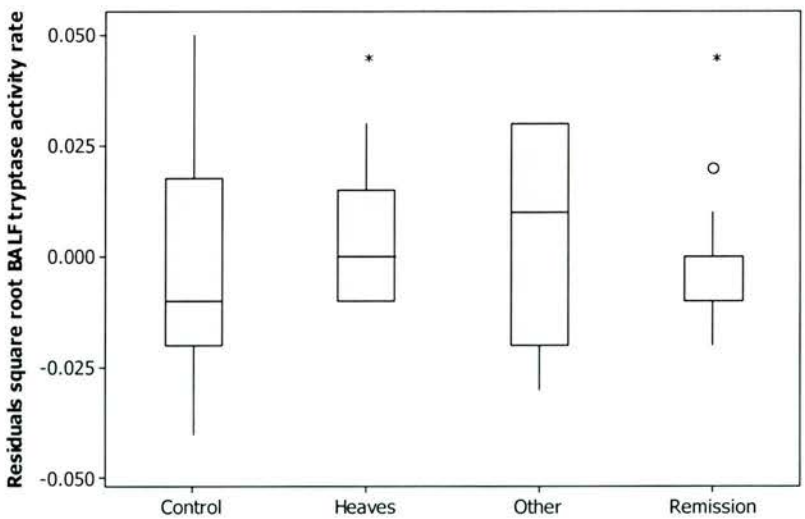

Fig. 4.24: BALF tryptase activity rates of residuals in control (n=20), heaves (n=17), other disease (n=7) and remission (n=11) horses following removal of the effect of time of sampling and place of challenge. * denotes significantly different groups (p<0.05).

Interestingly however, paired t-tests did not reveal a significant increase in BALF tryptase activity in the AHT archive subset of heaves susceptible horses following challenge (t=-0.8, p=0.50). Nor was there a significant difference in tryptase activity as a function of duration of challenge of heaves susceptible horses ($F_{1,16}$=1.0, p=0.33).

Regression analysis showed there was a very weak relationship between BALF tryptase concentration and BALF tryptase activity rate (t=1.55, p=0.13, R^2=4.6%) (fig. 4.25). Likewise, significant relationships were not found within individual groups of horses.

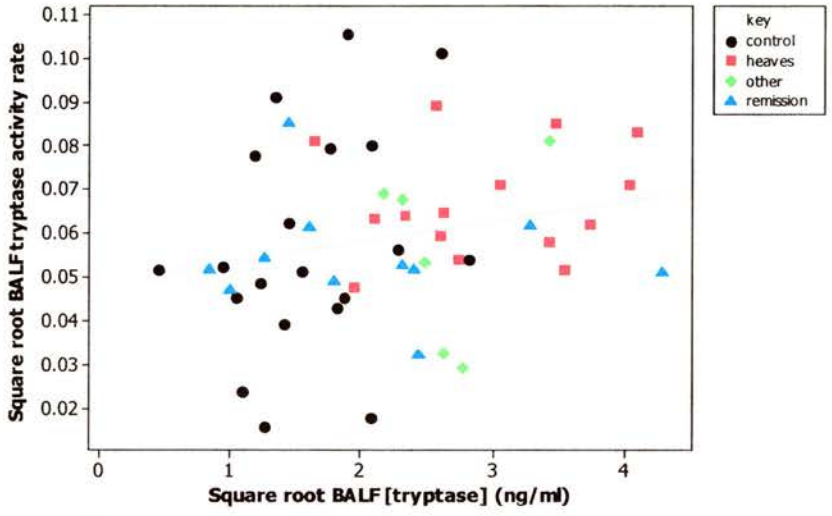

Fig. 4.25: Scatterplot demonstrating the weak positive relationship (t=1.55, p=0.13, R^2=4.6%) between BALF tryptase concentration and BALF tryptase activity rate. The grey line is a regression line for the whole dataset.

Furthermore, there was no relationship between BALF absolute mast cell count and tryptase activity rate (t=0.35, p=0.73, R^2=0.5%).

4.4.7. Western Blotting of BALF Samples

4.4.7.1. Western Blots for Probing of Mast Cell Proteinases

Spiked BALF samples and the positive control proteinase samples developed positive bands of approximately 32kDa for tryptase and eq.MCP-1 (figs. 4.26, 4.27) whereas unspiked samples failed to develop positive bands.

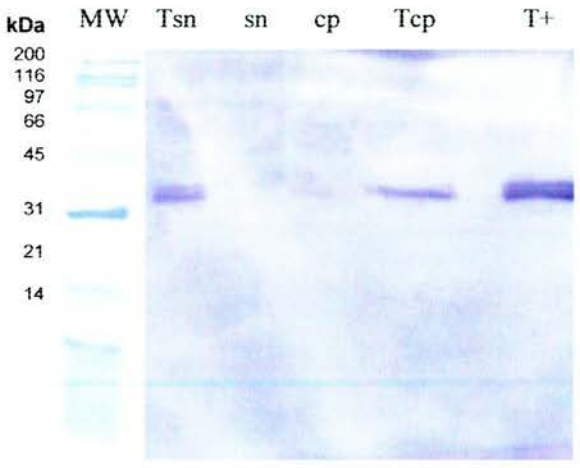

Fig. 4.26: Western blot of BALF supernatant and cell pellets, +/- addition of 0.1µg tryptase, probed with rabbit anti-equine tryptase. Positive bands of approximately 32kDa developed with tryptase spiked samples and the positive control (0.1µg) only. MW = molecular weight markers, Tsn = tryptase spiked supernatant, sn = supernatant, cp = cell pellet, Tcp = tryptase spiked cell pellet, T+ = positive control. kDa = size of molecular weight markers in kilo Daltons.

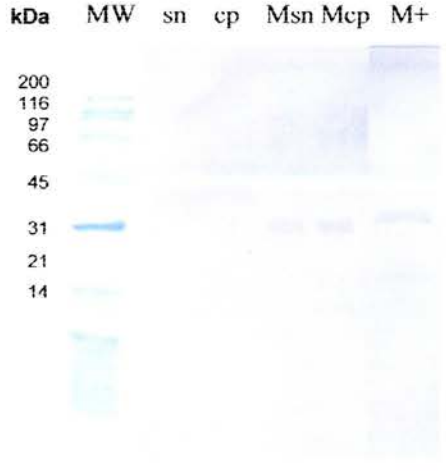

Fig. 4.27: Western blot of BALF supernatant and cell pellets, +/- addition of 0.1µg eq.MCP-1, probed with rabbit anti-eq.MCP-1. Positive bands of approximately 32kDa developed with eq.MCP-1 spiked samples and the positive control (0.1µg) only. MW = molecular weight markers, sn = supernatant, cp = cell pellet, Msn = eq.MCP-1 spiked supernatant, Mcp = eq.MCP-1 spiked cell pellet, M+ = positive control. kDa = size of molecular weight markers in kilo Daltons.

4.4.7.2. Western Blot to Determine Limit of Sensitivity of Mast Cell Proteinase Detection

The western blot probed with rabbit anti-equine tryptase in order to determine the limit of sensitivity for detection of mast cell proteinases did not develop any positive

staining with the cell pellet sample containing >200ng/ml tryptase. Cell pellet samples spiked with 1000ng and 500ng tryptase (100 and 50µg/ml) tryptase developed strong positive bands. Faint positive bands could be visualised with spiked tryptase samples down to 10ng tryptase (1µg/ml) (fig. 4.28).

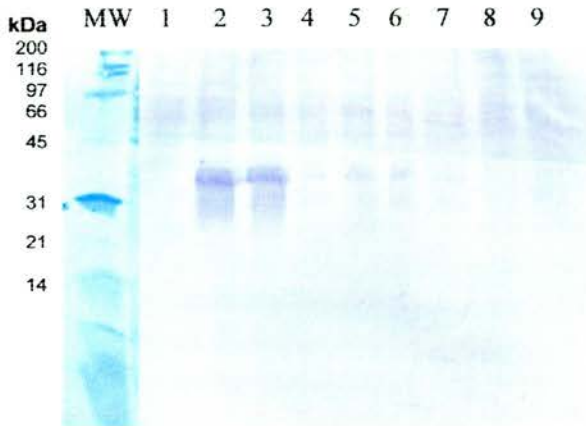

Fig. 4.28: Western blot of BALF cell pellet spiked with 0-1000ng tryptase probed with rabbit anti-equine tryptase. Strong positive bands developed for 1000 and 500ng tryptase and faint bands for 100, 50 and 10ng. A distinct band could not be discerned with tryptase spikes < 10ng. 1 = BALF cell pellet, 2-9 = BALF cell pellet spiked with 1000, 500, 100, 50, 10, 5, 1, 0.5ng tryptase respectively. kDa = size of molecular weight markers in kilo Daltons.

4.4.8. BALF Cytospin Immunolabelling

4.4.8.1. Trial Immunohistochemical Staining

No positive staining was seen with immunohistochemical staining using rabbit anti-equine tryptase / anti-eq.MCP-1 or mouse monoclonal anti-human tryptase-biotin (clone AA5). Antigen retrieval with 0.1mg/ml trypsin incubation did not improve staining.

4.4.8.2. Initial Immunofluorescent Labelling Protocol

Good labelling of mast cells was seen under 488nm excitation with rabbit anti-equine tryptase / anti-eq.MCP-1 (fig. 4.29a). However, some positively labelled cells were noted to have multi-lobed nuclei. Using autofluorescence of their nuclei, these cells with multi-lobed nuclei were determined to be neutrophils (fig. 4.29b). Occasional tryptase positive macrophages were also observed with this technique. The majority of positively labelled phagocytes had a granular appearance of labelling (fig. 4.29b) although a surface halo type appearance was seen less commonly (fig. 4.31a).

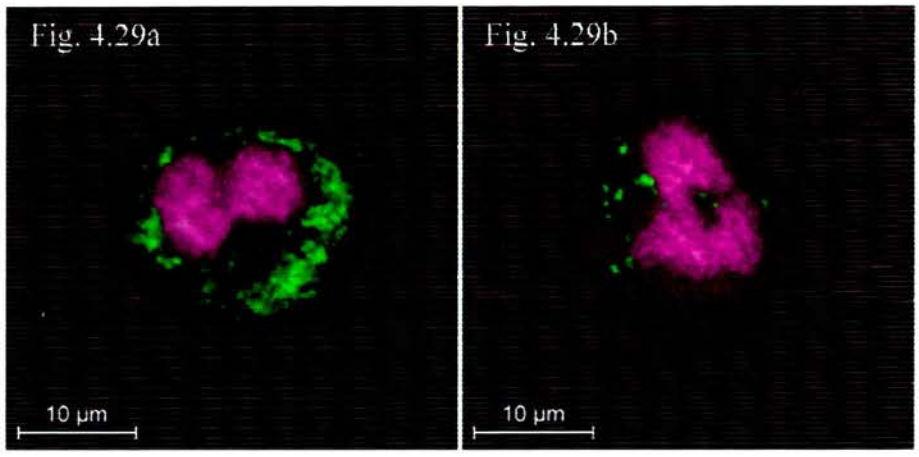

Fig 4.29: BALF mast cell (a) and neutrophil (b) showing positive labelling with rabbit anti-equine tryptase under 488nm excitation. Note the autofluorescence of the nucleus allowing cellular identification.

4.4.8.3. Western Blot to Investigate Possible Cross Reactivity with Rabbit Anti-Equine Tryptase

Probing of the western blot of equine tryptase, trypsin, equine elastase and eq.MCP-1 with rabbit anti-equine tryptase resulted in a positive reaction with equine tryptase only, demonstrating that rabbit anti-equine tryptase was specific for equine tryptase and does not show cross reactivity with the other proteinases tested (fig. 4.30).

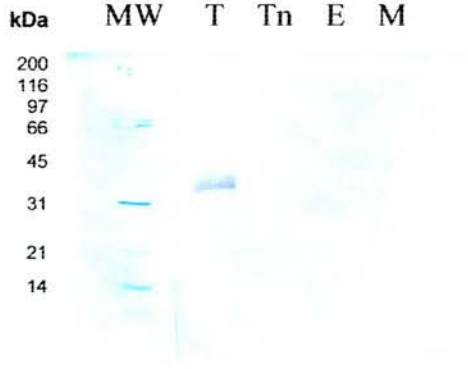

Fig. 4.30: Western blot of equine tryptase (T), trypsin (Tn), equine elastase (E) and eq.MCP-1 (M) (1µg/lane) probed with rabbit anti-equine tryptase. A positive reaction was only observed with tryptase. kDa = size of molecular weight markers in kilo Daltons.

4.4.8.4. Immunofluorescence of Peripheral Blood Neutrophils

Some immunofluorescent labelling of peripheral blood neutrophils was observed with rabbit anti-equine tryptase, although the majority of neutrophils were not labelled. Labelling of peripheral blood neutrophils was of a surface halo nature only, and thus could be readily differentiated from mast cells.

4.4.8.5. Western Blot of Peripheral Blood Neutrophils

Probing of the western blot of equine peripheral blood neutrophils with rabbit anti-equine tryptase resulted in the development of a very faint band with a molecular weight much higher than that of tryptase at approximately 67kDa, which was considered most likely to be non-specific reaction with albumin.

4.4.8.6. Final Immunofluorescent Labelling Protocol

Good labelling was achieved with the dual immunofluorescent protocol for tryptase (fig. 4.31) and the single labelling method for eq.MCP-1.

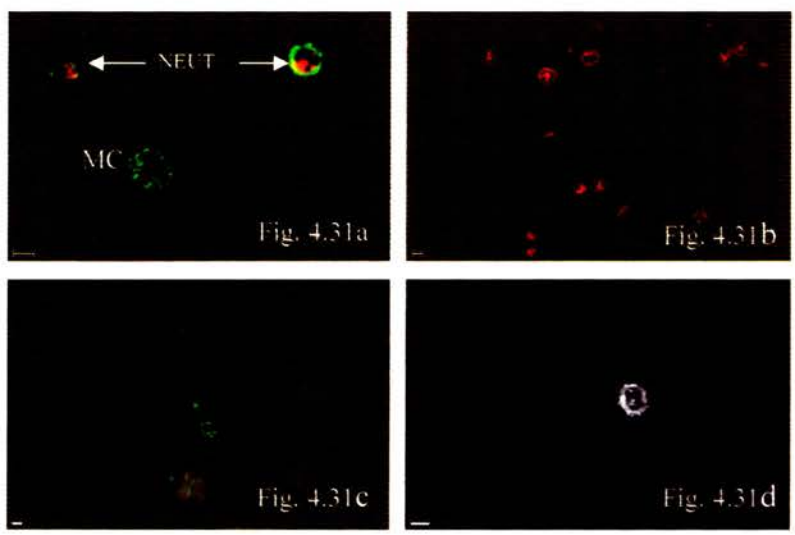

Fig. 4.31: Immunofluorescence of BALF cytospins: (a) positive labelling of a mast cell (MC) with rabbit anti-equine tryptase (green) and dual staining of neutrophils (NEUT) with rabbit anti-equine tryptase and sheep anti-equine neutrophil elastase (red) showing halo appearance of labelling, (b) tryptase negative control showing positive staining of neutrophils with sheep anti-equine neutrophil elastase, (c) elastase negative control showing positive staining of neutrophils with rabbit anti-equine tryptase, (d) eq.MCP-1 positive staining mast cell. Size markers all 10μm.

Data for the number of tryptase positive BALF mast cells could not be transformed to assume a normal distribution by either square root or log manipulation and was therefore analysed by Kruskal Wallis test. As the proportion of tryptase positive cells consisted of proportional data with values at both extremes of the scale, this was also analysed by Kruskal Wallis test. There was no significant inter-group difference in the number or proportion of tryptase positive mast cells ($p=0.89$ and $p=0.51$ respectively).

Statistical analysis of eq.MCP-1 data was not performed as eq.MCP-1 positive cells were so rarely observed. Eq.MCP-1 positive BALF cells were only observed in 6/19 (32%) horses compared to 18/19 (95%) horses with tryptase positive cells. The horses with observed eq.MCP-1 positive cells were distributed across the 3 status groups.

The number of elastase positive cells was log transformed such that the data met the assumptions for ANOVA. There was a significant inter-group difference in the number of elastase positive BALF cells ($F_{2,16}=0.74$, $p=0.02$). A two-sample t-test showed significantly increased elastase positive BALF cells in heaves horses compared to controls ($t=-3.6$, $p=0.006$).

4.5. Discussion

4.5.1. Development and Validation of ELISAs

Polyclonal rabbit anti-equine tryptase and mouse monoclonal anti-human tryptase-biotin (clone AA5) both recognised equine tryptase. However, when used in a sandwich ELISA together, no reaction developed, probably because the two antibodies shared a common epitope. As this mouse monoclonal antibody was only available biotinylated, the ELISA could not be performed using the monoclonal antibody as the coating antibody, which may have allowed the polyclonal antibody to bind to a different epitope. An alternative non-biotinylated mouse monoclonal anti-

human tryptase antibody was used; however this was from a different clone (AA1 rather than AA5) and appeared to have no specificity for equine tryptase.

Control experiments showed that there was very little non-specific background colour attributable to the other reagents used in both the tryptase and eq.MCP-1 ELISAs. Heparin-agarose treatment to remove tryptase resulted in an approximately equal reduction in tryptase concentration, as determined by the ELISA, from both spiked saline and BALF samples, confirming that the ELISA was specific for tryptase.

Determination of tryptase concentrations by the ELISA appeared to be more reliable at lower tryptase concentrations which did not require diluting to reach the standard curve. This, and the relatively high background colour that could not be improved by addition of a blocking step or by attempts to remove mucus using DTT treatment or centrifugation and filtration, will have led to some inherent inaccuracy in tryptase measurement, particularly in BALF samples with higher tryptase concentrations. BALF, being a biological sample, contains many molecules that could potentially interfere with the ELISA. It was suspected that mucus in particular might interfere because BALF from heaves horses (which also has increased tryptase concentrations) contains more mucus than that from controls (Dixon *et al.*, 1995b). DTT treatment of BALF samples markedly reduced the tryptase concentrations read by the ELISA and it was suspected that the DTT may have inactivated the coating antibody. Successful treatment of human sputum mucus with 0.01% DTT has been reported without a deleterious effect on subsequent ELISA measurement of tryptase (Pizzichini *et al.,* 1996; Alvarez *et al.,* 2000). However, sputum contains much more mucus than BALF and many studies have successfully measured human BALF tryptase by ELISA without DTT treatment (Wenzel *et al.,* 1988; Sedgwick *et al.,* 1991; Broide *et al.,* 1991; Jarjour *et al.,* 1991; Bousquet *et al.,* 1991). High speed centrifugation and filtration should have decreased the mucus content of samples. Therefore, as these procedures did not improve tryptase determination by the ELISA, it suggests that other BALF components may have been responsible for the

background colouration. This error in measurement due to background colouration is likely to have contributed to the four very high outlier tryptase concentrations.

4.5.2. BALF Tryptase Concentrations

The graphs of raw, square root and residual BALF tryptase concentrations are all qualitatively similar and demonstrate that BALF tryptase concentrations are higher in heaves horses than controls or heaves horses in remission. This increase was still highly significant ($p<0.001$) for heaves vs. controls and significant ($p<0.05$) for heaves vs. remission horses following removal of the effects of time of sampling and place of challenge. There was no significant difference in BALF tryptase concentrations between controls or challenged controls or between controls and heaves horses in remission. These results indicate that hay / straw challenge of control and heaves susceptible individuals causes tryptase release into the airway lumen in heaves susceptible horses only. It is therefore possible that tryptase could contribute to the pathogenesis of heaves as discussed in detail in section 1.4.4.1.

This study is the first to report equine BALF tryptase concentrations. Mast cell activation and tryptase release into BALF is well documented following challenge of human asthmatics (Wenzel *et al.,* 1988; Broide *et al.,* 1990; Broide *et al.,* 1991; Jarjour *et al.,* 1991; Bousquet *et al.,* 1991) with concentrations reported being similar to those found in this study (table 4.12). Comparison of values in horses to those in asthmatic subjects is not straightforward however due to the multiple human asthma phenotypes, including atopic and non-atopic asthma. Although BALF tryptase concentrations increase in both these asthma phenotypes, the magnitude of increase is greater in atopic asthmatics (Wenzel *et al.*, 1988). Control horses have considerably higher BALF tryptase concentrations than control human subjects which may reflect the higher equine intraluminal mast cell population. Control horses frequently have up to 10% mast cells in BALF (Dixon *et al.*, 1995b) compared to <1% in humans (Wenzel *et al.*, 1988; Broide *et al.*, 1991).

Group	**Mean (± SD) BALF [tryptase] (ng/ml)**
Clinical heaves	8.3 (4.4)
Remission heaves	5.1 (4.8)
Control horses	3.1 (2.0)
Symptomatic human asthmatics	10.1 (8.2)[1] 13.2 (14.8)[2]
Asymptomatic human asthmatics	2.0 (1.7)[1] 3.9 (3.9)[2]
Healthy humans	0.6 (0.4)[1]

Table 4.12: BALF tryptase concentrations (ng/ml) in control and heaves susceptible horses and asthmatic and healthy humans. [1](Wenzel *et al.,* 1988), [2](Broide *et al.,* 1991).

A highly significant positive correlation (Pearson $r=0.53$, $p<0.001$) was found between BALF tryptase concentration and severity of pulmonary inflammation as measured by BALF absolute neutrophil count. BALF neutrophilic response to *in vivo* challenge with hay dust suspension has previously been shown to be dose dependent (Pirie *et al.,* 2002a) and therefore the positive correlation between absolute neutrophil count and BALF tryptase concentration may suggest that there is a relationship between severity of inhalational dust challenge and BALF tryptase concentration. However it is difficult to examine this relationship fully due to the differences between the three challenges and the lack of a common specific measure of severity of challenge. Level of dust mite exposure is significantly correlated with sputum tryptase concentrations in asthmatics (Alvarez *et al.*, 2000) and ideally dust analysis or pulmonary function indices would have been used in this study to compare response to challenge; however this information was not available for all challenges.

There was no significant difference in tryptase concentrations between naturally and experimentally challenged animals, suggesting that experimental challenge was a reasonable model of the natural challenge environment. Duration and place of challenge and time of sampling post challenge had significant effects on BALF tryptase concentrations, demonstrating differences within the three challenge systems

and highlighting the difficulty in comparing heaves studies performed using different challenge models. It is unlikely that the difference in BALF tryptase concentrations in the different challenge systems (AHT archive, R[D]SVS archive and R[D]SVS hospital) can be attributed solely to duration of challenge, since there was a non-linear trend in tryptase concentrations with increasing duration of challenge. The source and composition of hay dust in the challenge environment is likely to have had an important influence on the response to challenge, since inhalation of the same quantity of hay dust from three different sources of hay resulted in markedly different responses in heaves susceptible horses (Pirie *et al.,* 2002a). It is likely that there was variation in organic dust composition among the three challenge systems which were from three different geographical areas. Interestingly, BALF tryptase concentration, but not BALF neutrophil count, was significantly affected by place of challenge. BALF tryptase concentration may therefore be a more sensitive indicator of pulmonary inflammation than neutrophil count. An *in vivo* dose response study investigating BALF tryptase concentrations following increasing organic dust challenge would need to be performed to clarify this relationship further.

Surprisingly, a significant correlation between BALF absolute mast cell count and tryptase concentration was not evident. Indeed, the increased BALF tryptase noted in heaves and other disease horses occurred despite a lack of increase in BALF mast cell numbers. Previous studies have also reported no increase in BALF mast cell ratios in heaves horses following hay / straw challenge (Dixon, Railton, & McGorum, 1995b; McGorum, Dixon, & Halliwell, 1993d). This is also consistent with the increased BALF tryptase concentrations reported in human asthmatics, without concurrent increase in BALF mast cells (Wardlaw *et al.,* 1986; Wenzel *et al.,* 1988; Chan-Yeung *et al.,* 1989; Broide *et al.,* 1991). This suggests that an increase in the number of BALF mast cells is not required to produce increased levels of BALF tryptase or alternatively, that mast cells outwith the BALF compartment, in particular those in an intra-epithelial location, may also be a source of BALF tryptase. Consistent with this possibility, increased numbers of partially and fully degranulated intra-epithelial mast cells have been identified in the bronchioles of human asthmatics compared to control subjects (Beasley *et al.,* 1989; Djukanovic *et al.,*

1990; Pesci *et al.,* 1993; Laitinen *et al.,* 1993; Di Stefano *et al.,* 1993; Carroll *et al.,* 2002a). This hypothesis is explored further in chapter 5.

Despite there being no significant difference in BALF tryptase concentrations between controls and challenged controls in the whole data set, paired data from the AHT archive study showed a significant increase in tryptase concentration in both the control and heaves susceptible horses following experimental challenge. Interestingly, these control horses also had a strong trend towards increased BALF absolute neutrophil count post challenge ($p=0.059$). It is well documented that control horses may develop a moderate BALF neutrophilia following severe challenge (Derksen *et al.,* 1985; Tremblay *et al.,* 1993; Pirie *et al.,* 2002a), although much lower than that seen in heaves susceptible horses. However, unlike heaves susceptible individuals, airway dysfunction and mucus hypersecretion do not occur (Pirie *et al.,* 2002a). It is possible that the control subgroup used in the AHT archive study received a sufficient challenge to cause an increase in BALF neutrophil count and tryptase concentration whereas the other control horses did not. However, the AHT subgroup of horses also had lower BALF tryptase concentrations following hay / straw exposure than the other groups, suggesting that they may have received a less severe challenge. As stated previously, as the source and composition of dust is an important determinant of the challenge response, this may explain the different response seen in the AHT group of horses. The tryptase concentrations of the R(D)SVS archive and R(D)SVS hospital populations share greater similarity, suggesting that the R(D)SVS experimental challenge may have mimicked natural hay / straw challenge more closely than the AHT model.

Horses with other pulmonary diseases also had significantly increased BALF tryptase concentrations compared to controls suggesting that elevated BALF tryptase concentrations are not specific for heaves and may occur with other diseases with a pulmonary inflammatory response. This group was comprised predominantly of horses with putative (unconfirmed) viral respiratory infections (4/7 samples), which have previously been reported to result in increased BALF tryptase concentrations in other species (Lorente *et al.,* 2001; Jolly *et al.,* 2004). Indeed some studies have

suggested that tryptase can activate respiratory viruses such as influenza (Chen *et al.,* 2000; Lorente *et al.,* 2001; Sato *et al.,* 2003). One sample in this group was collected from a horse with inflammatory airway disease (IAD), which shares some clinical and pathological features of heaves (Hoffman *et al.*, 1998; Couetiel *et al.*, 2001). Furthermore, increased BALF mast cells have been reported in some cases of IAD (Hoffman *et al.*, 1998) and it therefore seems probable that tryptase release may also occur with this disease.

4.5.3. BALF Eq.MCP-1 Concentrations

Very little eq.MCP-1 was present in the BALF from any of the groups of horses; with over half of the samples having no detectable eq.MCP-1. BALF chymase concentrations have not previously been reported in horses or other species. It would appear that basal BALF eq.MCP-1 concentrations are low and that, in contrast to tryptase, eq.MCP-1 is not released following challenge of heaves susceptible individuals. As samples were harvested from both acute and chronically challenged horses at different timepoints, it seems unlikely that elevated eq.MCP-1 BALF concentrations were missed due to insufficient sampling. This is further supported by the paucity of eq.MCP-1 labelled cells present in BALF and in airway epithelium from control and heaves horses (sections 4.4.8 and 5.5.1). Collectively, these findings suggest that eq.MCP-1 may be of little importance in the pulmonary airspace of healthy horses and horses with the types of pulmonary inflammation studied. In other species, chymases appear to be important in expulsion of nematodes (Knight *et al.,* 2002) and this can not be excluded in the equine lung as no horses with lungworm were sampled.

4.5.4. BALF Histamine Concentrations

Histamine was undetectable in two thirds of BALF samples and displayed a wide range of concentrations (0.1-7.5ng/ml) in the remaining samples. A likely explanation for this observation appears to be degradation of labile histamine in some samples. This was evident in some of the R(D)SVS hospital samples and to a greater extent in archived samples which had been stored for longer periods of time

and which may have undergone prior freeze-thawing. Although samples were acylated as a stabilising step prior to testing on the commercial immunoassay as recommended, it appears that prior histamine degradation had occurred in some samples. This explanation seems likely given that samples from the R(D)SVS hospital population, which had been stored for a shorter period and had not undergone repeated freeze-thawing prior to analysis, had significantly greater histamine concentrations than the archived samples. McGorum *et al.* (1993c) found only minor changes in histamine concentration of BALF supernatant samples during prolonged (but unspecified duration) storage at -20°C, however storage of whole BALF samples on ice for up to 1h prior to collection of supernatant resulted in a progressive decline in histamine concentration with a half life of 15-50min. If samples are not stored on ice, the half life of histamine is reported to be as short as 1min (Keyzer *et al.*, 1984). Although supernatant was separated from all BALF samples within 15min of collection, histaminase activity would still be present in the supernatant and degradation may have occurred during centrifugal concentration of BALF supernatant samples which was performed prior to acylation over 90min at 4°C. Furthermore, archived samples may have also previously been thawed for unknown periods of time at unknown temperatures. Although studies have reported human (Rankin *et al.*, 1987) and equine (Hare *et al.*, 1994) BALF histamine concentrations to be stable for over 2 months at -70°C, the duration of recommended storage may have been exceeded as archived samples were stored for more than a year. Interestingly, if diluted plasma samples are frozen prior to histamine analysis, there is a dramatic decrease in histamine such that it is recommended that diluted samples should be alkylated (to stabilise histamine) prior to freezing if they cannot be analysed immediately (Laroche *et al.*, 1995). This loss of histamine by freezing diluted samples may also have occurred with the BALF samples.

Histamine appears to be less useful than tryptase as a marker of mast cell degranulation due to its labile nature. Furthermore, tryptase concentrations have been found to correlate more closely with clinical symptoms (Castells and Schwartz, 1988) and antigen challenge dose (Sedgwick *et al.*, 1991) of hay fever sufferers than histamine concentrations.

It has been well documented that BALF histamine concentrations in asymptomatic and symptomatic human asthmatics are significantly greater than non-asthmatic controls (Casale *et al.,* 1987b; Wenzel *et al.,* 1988; Casolaro *et al.,* 1989; Chan-Yeung *et al.,* 1989; Broide *et al.,* 1991; Jarjour *et al.,* 1991). Although mean and median BALF histamine concentrations were greatest in heaves horses in the hospital data subset of this study, no statistical difference in histamine concentrations was observed among groups due to the wide range in data, which included nine samples with undetectable (and possibly degraded) histamine. Therefore, the high proportion of histamine negative samples may have reduced the power to detect a difference between control and heaves horses.

To the author's knowledge, the only prior study measuring equine BALF histamine concentrations is that of McGorum *et al.* (1993c) which detected increased PELF histamine concentrations in heaves susceptible horses 5h following hay / straw challenge, however BALF concentrations were not significantly increased. Due to the variable dilution of retrieved PELF by lavage fluid, PELF concentrations of a given constituent may be more accurate than BALF concentrations; however urea and albumin standardisation methods (the two most frequently used) may lead to either under or over-estimation of concentrations (Rennard *et al.,* 1986; Marcy *et al.,* 1987). Furthermore, if a standard lavage volume is instilled (as occurred with the R[D]SVS hospital population), it is reasonable to assume that released histamine is homogenously diluted such that recovered BALF yields a representative aliquot reflecting histamine concentration in total BALF. Samples necessary to calculate PELF constituent concentrations (i.e. paired BALF and serum samples) were not collected for the archived samples and could not be collected for hospital samples and therefore it was not possible to calculate PELF histamine concentrations in this study.

BALF histamine concentrations for the hospital subgroup of horses reported in this study are similar to those previously reported in horses by McGorum *et al.* (1993c)

or in human asthmatics (Wenzel *et al.,* 1988; Chan-Yeung *et al.,* 1989; Broide *et al.,* 1991; Jarjour *et al.,* 1991) (table 4.13).

Group	Mean, median (horses only) (± SD) BALF [histamine] (ng/ml)
Clinical heaves	2.5, 1.9 (2.9)[1] 1.1 (0.02-6.4)[2] *
Remission heaves	1.5, 0.2 (3.0)[1]
Control horses	0.6, 0.1 (1.4)[1] 1.4 (0.1-2.4)[2] *
Challenged human asthmatics	2.8 (2.0)[3] 4.8 (5.0)[4] 3.7 (2.6)[5] 0.2 (0.2)[6]
Asymptomatic human asthmatics	0.7 (1.1)[3] 0.2 (0.2)[4] 2.3 (2.3)[5]
Healthy humans	0.2 (0.2)[3] 0.2 (0.1)[4] 0.04 (0.006)[6]

Table 4.13: BALF histamine concentrations (ng/ml) in control and heaves susceptible horses and asthmatic and healthy humans. [1]current study (R[D]SVS hospital population only), [2]McGorum, Dixon and Halliwell (1993c), [3]Wenzel, Fowler and Schwartz (1988), [4]Broide *et al.* (1991), [5]Chan-Yeung *et al.* (1989), [6]Jarjour *et al.* (1991). * Median and range only reported by authors.

Histamine concentrations have been reported to increase in human blood and plasma if not stored on ice due to an inherent 'leakiness' of basophils which is more apparent at higher temperatures (Laroche *et al.*, 1995). Leakage of histamine from mast cells should not have occurred in this study because BALF was stored on ice until separation of supernatant within 15min of collection. Bacteria have also been shown to cause histamine release from human BALF mast cells (Clementsen *et al.*, 1991). Although microbiological culture was not performed on BALF samples, it is unlikely that significant bacterial contamination occurred due to the speed of harvesting supernatant from cells.

Only a weak correlation was evident between BALF histamine and tryptase concentrations. Although it might be expected that constituents of the same cell would be highly correlated, there is evidence to suggest that differential degranulation of mast cell mediators can occur (Dvorak *et al.*, 1996; Theoharides, 2002). However, data available to enable investigation of a potential relationship between these two variables was limited to the R(D)SVS hospital data subset due to the high number of (presumed) degraded samples in the other data subsets. Histamine and tryptase concentrations are positively correlated in human asthmatics (Wenzel *et al.*, 1988; Broide *et al.*, 1991) and a stronger relationship is likely to have been found if histamine concentrations were available for more samples. BALF histamine concentrations have also been shown to closely correlate with lung function indices in asthmatics (Flint *et al.*, 1985a; Casale *et al.*, 1987a; Wardlaw *et al.*, 1988; Broide *et al.*, 1991; Jarjour *et al.*, 1991), however these comparisons could not be performed in this study as lung function was not measured in horses referred to R(D)SVS hospital and since archived samples, for which lung function data was available, had undetectable histamine concentrations.

4.5.5. Tryptase Activity

A significant difference in BALF tryptase-like activity was found among groups. When potential confounding factors had first been taken into consideration, BALF from heaves horses still contained significantly greater tryptase-like activity than that from remission horses. Similarly, Clarke *et al.* (1995) showed that antigen challenge of allergic sheep increased BALF tryptase activity and could be blocked by the tryptase inhibitor APC 366. Interestingly however, despite both control and heaves susceptible horses from the AHT archive group having a significant increase in BALF tryptase concentration post challenge, neither group showed a significant increase in apparent tryptase activity. The AHT subgroup of horses had the lowest tryptase concentrations post challenge, which may not have been sufficient to measure an increase in BALF tryptase activity.

Although the mean and median tryptase activity rate of control BALF samples (0.0035, 0.0026 absorbance units/h [AU/h], respectively) was lower than those of the heaves affected group (0.0049, 0.0042 AU/h, respectively), the range of activity rates from the control group (-0.0004 to 0.0112 AU/h) was very wide, such that it is not surprising that there was no significant difference between controls and heaves affected horses.

Tryptase activity was measurable at concentrations found in equine BALF. Tryptase activity rates of BALF samples showed only a weak relationship with tryptase concentrations determined by the ELISA and there could be several explanations for this finding. For example, the presence of additional trypsin-like proteinases in the BALF may have contributed to the activity rate and therefore the apparent active tryptase concentration. Additionally, tryptase present in samples was subject to an unknown degree of dissociation into inactive monomers, by the action of heparin scavengers such as neutrophil lactoferrin, which would add to the scatter of results. Lavens *et al.* (1993) measured tryptase activity from human lung mast cells over a 72h period using benzoyl-DL-arginine-p-nitroaniline (BAPNA) and found good correlation between tryptase activity and tryptase concentration. That study however assessed tryptase immediately following mast cell stimulated release by degranulating agents and also did not assess tryptase activity in complex biological samples. BAPNA was not used in this study due to its very poor cleavage by equine tryptase (Pemberton *et al.*, 2001).

4.5.6. Western Blot of BALF Samples

Tryptase could be detected on probing of western blots with rabbit anti-equine tryptase down to 1µg/ml. As even BALF supernatant and cell pellet samples with high concentrations of tryptase contained less than 1µg/ml tryptase, it is not surprising that these samples did not develop visible positive bands. Probing of western blots was therefore not sufficiently sensitive to demonstrate mast cell proteinase content of equine BALF samples.

4.5.7. BALF Cytospin Immunolabelling

4.5.7.1. Positive Tryptase Labelling of Non-Mast Cells

The positive labelling of BALF neutrophils and occasional macrophages with rabbit anti-equine tryptase may have been due to recognition of phagocytosed degranulated tryptase, or even whole mast cells, by these phagocytic cells. As it has now been demonstrated that equine BALF contains tryptase, it seems probable that some of this would be engulfed or otherwise scavenged by phagocytic cells as has been described with neutrophil elastase (Schmekel *et al.*, 1990), in a control mechanism to limit proteolysis in the airway. Tryptase positive macrophages have previously been reported in human BALF and lung tissue and their presence attributed to phagocytosis of tryptase (Walls *et al.,* 1990; Berger *et al.,* 1999; Beil and Pammer, 2001).

Occasional peripheral blood neutrophils also labelled positively with rabbit anti-equine tryptase but it seems unlikely that tryptase would be phagocytosed in the bloodstream. Compared to the granular nature of labelling of the majority of BALF neutrophils, immunolabelling was restricted predominantly to the surface of peripheral blood neutrophils. It would seem extremely unlikely that BALF neutrophils and macrophages contain endogenous tryptase since significant expression of tryptase has only ever been described in mast cells. Although basophils contain tryptase, it is only at approximately 0.4% of the concentration observed in mast cells, which is too low to stain positively with immunohistochemistry (Castells *et al.*, 1987). Recently, human airway epithelial cells have been reported to contain a serine proteinase termed tryptase epsilon, however this proteinase has only 38-44% sequence homology with other human tryptases and therefore is likely to be different in structure and substrate specificity to mast cell tryptase (Wong *et al.*, 2001). It is feasible that due to the surface nature of labelling observed in peripheral blood neutrophils, a trans-membrane tryptase is also present on these cells and that rabbit anti-equine tryptase showed low specificity to this molecule. However, the faint band on probing the western blot of peripheral blood neutrophils was of higher molecular

weight than tryptase or trans-membrane tryptase and was considered to be most likely due to non-specific reaction with albumin.

Alternatively, non-specific binding may have occurred with the positive labelling resulting from cross-reactivity of the polyclonal rabbit anti-equine tryptase with a constituent of the other cells. Unfortunately monoclonal equine tryptase antibodies, which would have been more specific, were not available for use. Cross-reactivity with equine neutrophil elastase was excluded by western blot analysis and furthermore, dual immunofluorescence demonstrated that elastase and tryptase labelling did not co-localise. However cross-reactivity of the polyclonal rabbit anti-equine tryptase with another neutrophil or macrophage protein is possible.

In summary, labelling of BALF phagocytes was considered most likely to be due to positive labelling of phagocytosed tryptase granules due to the granular nature of labelling and the lack of positive phagocyte labelling in other tissues tested (colon and lung, section 2.5.3.3). Furthermore, no non-phagocytic cells stained positively with the anti-equine tryptase in BALF. In contrast, it was considered that labelling of peripheral blood neutrophils was most likely a result of non-specific cross-reaction to a surface protein due to the surface nature of labelling. However, the cause of positive phagocyte labelling could not be resolved fully.

4.5.7.2. Immunofluorescence of Mast Cells

Tryptase positive mast cells were the predominant mast cell phenotype in equine BALF, with only occasional chymase positive cells, as documented in humans (Flint *et al.*, 1985b; Wenzel *et al.*, 1988).

There was no significant difference in the number or proportion of tryptase positive BALF mast cells between control and heaves horses and this can be interpreted in several ways. It most likely suggests that there is no recruitment of tryptase positive BALF mast cells to the airway lumen supporting the hypothesis that intra-epithelial mast cells are an important source of BALF tryptase. It also appears that BALF mast cells of heaves susceptible horses do not 'switch on' tryptase expression following

challenge, as the proportion of tryptase positive mast cells is not increased in heaves horses. These results may also suggest that BALF mast cells do not fully degranulate whilst releasing tryptase into the BALF as this would have decreased the number of immunolabelled cells. However, it is also possible that additional influx of mast cells and full degranulation of mast cells already present occur in parallel such that whilst there is turnover of the mast cell population, the number of mast cells present in the airways appears to remain stable.

As so few eq.MCP-1 positive BALF mast cells were seen in all horses, it is not surprising that there was no significant difference in the number of immuno-positive cells between control and heaves affected horses.

Heaves affected horses had significantly increased numbers of elastase positive cells, reflecting the neutrophil influx into the airspace characteristic of clinical heaves.

4.6. Conclusion

This study has provided evidence for mast cell degranulation in heaves susceptible, but not control, horses following natural hay / straw exposure. Furthermore, BALF tryptase concentrations were significantly correlated with the severity of pulmonary inflammation suggesting that mast cells may play a pivotal role in the pathogenesis of the pulmonary inflammatory response in heaves. However, the role that tryptase, or indeed any of the many other mast cell mediators, may play in the pathogenesis of heaves remains to be elucidated. The source of BALF tryptase may be mast cells in the airway lumina, within the airway epithelium, or less likely within the parenchyma: this is explored further in Chapter 5. In contrast, BALF contained very little eq.MCP-1 suggesting that eq.MCP-1 is of little importance in the airway of healthy and heaves susceptible horses. Consistent with these findings, BALF mast cells were predominantly tryptase positive and only rarely eq.MCP-1 positive. Histamine appears to be a less stable, and therefore potentially a less useful indicator of mast cell degranulation in the horse.

Chapter 5 : Proteinase Expression in the Equine Lung in Response to Inhaled Dust Challenge

5.1. Summary

Mast cell proteinase expression in the equine lung was investigated at the protein and mRNA level in control and heaves susceptible horses, before and after inhaled dust challenge.

To investigate pulmonary mast cell distribution and phenotype, donated bronchial and bronchiolar samples from 6 control and 7 heaves horses in early resolution phase following dust challenge were stained with toluidine blue, anti-equine tryptase and anti-eq.MCP-1. Mast cells were enumerated in the epithelium, connective tissue, smooth muscle and alveoli and compared between control and heaves populations. The vast majority of mast cells were tryptase positive, with eq.MCP-1 positive cells rarely observed. There was a trend towards increased numbers of mast cells in the epithelium of heaves horses that became statistically significant following the exclusion of one control horse outlier.

To investigate pulmonary mast cell tryptase mRNA transcript expression in control and heaves horses, RNA was extracted from BALF cell pellets from control (n=3) and heaves susceptible (n=6) horses pre and post 48h hay / straw challenge and from the bronchial and bronchiolar samples described above. Quantitative real-time RT-PCR revealed no significant upregulation in BALF cell pellet tryptase mRNA expression in control or heaves susceptible horses following challenge. In contrast, bronchiolar tryptase transcripts were significantly down-regulated seven-fold in heaves horses in early resolution phase compared to control horses. Bronchial tryptase transcripts were not significantly different between control and heaves horses, consistent with the premise that heaves is predominantly a disease of the small airways.

This study supports the involvement of bronchiolar mast cells, particularly those in the epithelium, in the pulmonary inflammatory response in heaves horses. Therefore airway luminal mast cells may be a senescent population of cells which contribute little to the aforementioned (Chapter 4) increase in BALF tryptase noted in heaves horses post challenge.

5.2. Introduction

In Chapter 4 it was demonstrated that heaves horses have significantly increased BALF tryptase concentrations during clinical exacerbation compared with control horses or heaves horses in remission. Elevated BALF tryptase concentrations may be a result of degranulation of BALF mast cells and / or airway tissue mast cells. As challenge of heaves susceptible horses does not result in mast cell influx into the BALF compartment (Derksen *et al.,* 1985; McGorum *et al.,* 1993d; Dixon *et al.,* 1995b), it was hypothesised that recruitment and degranulation of airway epithelial mast cells may significantly contribute to this increase in BALF tryptase concentration. The observation of increased mast cells in the bronchial mucosa of occupational asthmatics would support this hypothesis (Di Stefano *et al.*, 1993). Furthermore, allergen challenge in sensitised asthmatics induces mast cell infiltration into the airway mucosa with subsequent degranulation (Casale *et al.,* 1987b; Pesci *et al.,* 1993; Montefort *et al.,* 1994).

The numbers of mast cells within airway smooth muscle are also elevated in human asthmatics compared to controls (Ammit *et al.*, 1997), where they are believed to contribute to bronchospasm through their action as both a direct agonist and mitogen for myocytes (Johnson *et al.*, 1997; Berger *et al.*, 2001a). Furthermore, mast cell mediators have been shown to augment cholinergic tone in the equine airway and have been suggested as important mediators of the mechanism for development of airway obstruction in heaves (Olszewski *et al.*, 1999).

Although mast cell distribution in the lungs of control horses and horses with chronic lung disease (of unknown aetiology) has been investigated (Nicholls, 1978; Mair *et*

al., 1988; Winder and von Fellenberg, 1990), mast cell proteinase content and distribution in control and confirmed cases of heaves have not been studied. It was hypothesised that mast cell recruitment to the epithelium and smooth muscle may occur in heaves susceptible horses following challenge, thus contributing to increased BALF tryptase concentrations and airway obstruction respectively.

We were also interested in investigating tryptase mRNA expression in both luminal and tissue mast cells from control and heaves susceptible horses in response to challenge. Although quantitative studies of cytokine regulation in BALF cell pellets have been performed with attention focused on T lymphocyte populations (Giguere *et al.,* 2002; Beadle *et al.,* 2002; Ainsworth *et al.,* 2003a; Ainsworth *et al.,* 2003b), there have been no studies investigating possible upregulation of proteinases or cytokine transcripts in equine airway luminal mast cells, using cell specific markers, during challenge of heaves susceptible horses. Equally, no studies have evaluated the equine pulmonary tissue mast cell response to challenge. Even though mast cell regulation has been explored in asthmatics and allergic rhinitis patients, these studies have focused on mast cell cytokine, rather than proteinase, regulation (Kay *et al.,* 1995; Jaffe *et al.,* 1995; Pawanker *et al.,* 2000).

The aims of this study were therefore: (a) to investigate the number, distribution and proteinase content of mast cells in the equine lung and (b) to probe tryptase mRNA transcript regulation in BALF cell pellets and lung tissue in control and heaves horses using quantitative real-time RT-PCR.

5.3. Materials and Methods

5.3.1. Collection of Tissues for Mast Cell Staining

Bronchial and bronchiolar tissue samples from control (n=6) and heaves susceptible horses (n=7) were donated from a study performed at Michigan State University. The controls comprised 3 mares and 3 geldings with a median (range) age of 18y (12-21y). The heaves susceptible horses comprised 2 mares and 5 geldings with a median (range) age of 21y (11-34y). Horses were subjected to a high dust challenge

environment (3.6m x 3.6m, poorly ventilated stable bedded with straw and fed poorly saved hay) for 5d, followed by 7d in a low dust environment where hay and straw were replaced with shavings and pelleted food. Bronchoalveolar lavage with cytological analysis of retrieved fluid was performed on day 7 of the low dust regimen. Heaves horses demonstrated a significant BALF neutrophilia in response to challenge compared with control horses indicating that they had ongoing pulmonary inflammation and were only in an early phase of resolution.

Horses were euthanased on day 8 of low dust regimen. Immediately post mortem, small (approximately 1cm x 1cm) pieces of tissue were excised from a peripheral area of lung and from a third generation bronchus and immersed in Carnoys fixative for subsequent mast cell staining. The peripheral lung tissue was examined grossly on cross section to ensure that several small bronchioles (<0.2cm) were present. After 24h in Carnoys fixative, tissues were transferred to 70% ethanol until processed further. Carnoys fixative is reported to be the preferred fixative for identification of mast cells as formalin fixation leads to underestimation of mucosal mast cells (Nicholls, 1978; Irani *et al.,* 1986; Craig *et al.,* 1986; Shanahan *et al.,* 1987; Winder and von Fellenberg, 1990; Kube *et al.,* 1998; Kuther *et al.,* 1998). Indeed, preliminary trials with 4% paraformaldehyde fixed tissues revealed very poor staining of mucosal mast cells, with toluidine blue in particular, which was not improved following employment of antigen retrieval techniques discussed in Chapter 2 (data not shown). Furthermore, a subsequent pilot study of toluidine blue staining in the lung following fixation with Carnoys fixative, 4% paraformaldehyde, zinc fixative and isoformal acetic acid confirmed Carnoys fixative to be the optimal fixative for histochemical enumeration of mast cells (data not shown).

5.3.2. Processing of Lung Samples

Samples were submitted to the Division of Veterinary Pathology at R(D)SVS for paraffin embedding, cutting of 4μm sections and mounting onto glass slides.

5.3.3. Mast Cell Staining

All staining was performed in duplicate.

5.3.3.1. Toluidine Blue Staining

Sections were de-waxed with xylene, rehydrated though graded alcohols and water and then immersed in 0.5% toluidine blue (Merck) in 0.5M HCl, pH 0.5, overnight. Sections were then briefly washed with tap water and counter-stained by immersion in 1% eosin (Surgipath) in 70% ethanol for 5s. Following a brief wash with tap water, slides were allowed to air-dry and coverslips applied with DPX mountant (Surgipath).

5.3.3.2. Proteinase Immunohistochemistry

Slides were de-waxed with xylene, rehydrated through graded alcohols and immersed in 97% methanol / 3% hydrogen peroxide for 20min to quench endogenous peroxidase activity. Slides were then transferred to Sequenza slide carriers (Shandon) and non-specific binding blocked with PBS / 0.5M NaCl / 0.5% Tween 80 (Sigma) / 10% normal horse serum for 30min. All further dilutions were made using this blocking buffer unless otherwise stated. Sections were incubated with 1μg/ml rabbit anti-equine tryptase / anti-eq.MCP-1 / normal rabbit IgG, for 60min, washed with PBS and then incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) at 1/400 dilution, for 30min. Following washing, slides were treated with avidin-horseradish peroxidase conjugate (ABC kit, Vector Laboratories) for 30min and then colour developed using 3,3'-diaminobenzidine (DAB kit, Vector Laboratories). Sections were then counterstained with Mayer's haematoxylin (Sigma), washed in Scott's tap water, dehydrated and mounted.

5.3.4. Mast Cell Counting

Slides were read at x250 magnification with a Leica microscope (Leica Laborlux S conventional compound microscope, Leica Microsystems). The number of positive cells in 20 views (10 per duplicate slide) of alveolar, bronchiolar and bronchial tissue was recorded. Bronchial and bronchiolar mast cells were identified as being intra-

epithelial, smooth muscle or connective tissue in location. Connective tissue was defined as mucosal (excluding epithelium) and submucosal tissue.

An image of each counted area was acquired with a Sony DXC-390P 3CCD colour video camera (Scion Corporation) to allow post-processing. The RGB video signal from the camera was digitised using Scion Image (Scion Corporation) installed on a G4 Macintosh computer (Apple Computer) fitted with a CG-7 frame grabber (Scion Corporation). Stereological measurements according to Gunderson and Jensen (1987) were performed using custom software developed for Object-Image (Vischer *et al.*, 1994). Object-Image is a public domain software package, based upon NIH image (Rasband and Bright, 1995), and is available via the internet at *http://simon.bio.uva.nl/object-image.html*. This allowed the area of alveolar, smooth muscle, connective tissue and epithelial tissue to be calculated excluding any air, such that the frequency of mast cell counts per mm^2 of tissue could be determined. Briefly, a grid was placed over the tissue image and each cross-point of gridlines was attributed one of the four tissue descriptions or air. The number of cross-points per tissue type was then expressed as a percentage of the total number of image cross-points to find the proportion of each tissue type examined. The image area was calculated and then the proportion of each tissue type used to calculate the area of that particular tissue observed per image. Mast cell counts and areas of tissues examined for the 20 fields of view were added together to give one mast cell count per location per bronchial or bronchiolar tissue per horse.

5.3.5. Collection of Samples for RNA Extraction

5.3.5.1. BALF Cell Pellets

Archived BALF cell pellets stored at -70°C in Qiagen RLT Buffer (RNeasy Mini Kit, Qiagen) were used to examine airway luminal mast cell tryptase transcript regulation in response to hay / straw challenge. BALF had previously been collected from control (n=3) and heaves susceptible (n=6) horses at baseline (-7d) and immediately post 48h hay / straw challenge as previously described (4.3.1.1). The control horses comprised 2 mares and one gelding with a median (range) age of 4y

(4-6y). The heaves susceptible horses comprised 4 mares and 2 geldings with a median (range) age of 14.5y (7-28y). Challenge conditions were as described in section 4.3.1.2, however airborne dust concentrations were not measured for this challenge. BALF cells were pelleted by centrifugation (400g, 10min), resuspended in RLT Buffer and stored at -70°C.

5.3.5.2. Lung Tissue Samples

Tissue samples were collected as described, and from the same horses as detailed in section 5.3.1. Harvested samples were stored in RNA later (Ambion) at 4°C overnight and then transferred to -20°C until processing. Inexplicably, samples from one control horse appeared to disintegrate in RNA later and therefore could not be used for RNA extraction.

5.3.6. RNA Extraction

Total RNA was extracted from lung tissue initially using TRI-reagent and chloroform and latterly with the RNeasy mini kit (Qiagen). BALF cell pellet total RNA was also extracted using this kit.

5.3.6.1. RNA Extraction Using TRI-reagent and Chloroform

RNA extraction and DNase treatment was performed as described in section 3.3.2.

5.3.6.2. RNA Extraction Using RNeasy Kit

Total RNA was extracted as per the manufacturer's instructions. Briefly, approximately 20mg lung tissue was homogenised with 600µl RLT buffer, the tissue lysate centrifuged 13,000g, for 3min and the supernatant harvested. BALF cell pellets in RLT buffer joined the protocol at this point.

Following addition of 600µl 70% ethanol, RNA was loaded onto the silica membrane of the RNeasy spin column by centrifugation at 13,000g, for 15s. RNA was washed with buffer RW1 and on-column DNA digestion performed by incubation with 80µl DNase I (RNase-Free DNase Set, Qiagen) for 15min, at room

temperature. DNA-free RNA was washed with buffers RW1 and RPE, eluted in 30µl RNase free water and the concentration and purity measured by spectrophotometry.

5.3.7. Reverse Transcription of RNA

DNA free mRNA was reverse transcribed using random hexamer primers and avian myeloblastosis virus (AMV) reverse transcriptase (Reverse Transcription System, Promega). The following reaction was set up on ice; 4µl 25mM $MgCl_2$, 2µl 10x reverse transcription buffer (100mM Tris HCl, pH 8.8), 2µl 10mM dNTPs, 0.5µl (20u) RNase inhibitor, 0.5µl (15u) AMV-RT, 1µl (0.5µg) random hexamers and 1µg RNA. The reaction mixture was incubated for 10min at room temperature, 50 min at 42°C and finally for 5min at 99°C to stop the reaction. The mixture was cooled on ice, diluted to 100µl with RNase free water and the cDNA concentration measured by spectrophotometry.

5.3.8. Quantitative RT-PCR

Primers were designed from the known cDNA sequences for equine tryptase (GenBank accession number AJ515902) and the house-keeping gene equine β-actin (GenBank accession number P60708) to give products of 191bp and 500bp respectively as indicated in table 5.1. Quantitative real-time RT-PCR was performed by amplifying 250ng cDNA (in a volume of 8µl) with 10µl SYBR Green Master Mix (Quantitect SYBR Green PCR Kit, Qiagen) and 1µl each of forward and reverse primers (6µM, final reaction concentration 0.3µM) in an Opticon DNA Engine (MJ Research). The reaction was incubated at 95°C for 15min, to activate the HotStarTaq DNA polymerase contained in the SYBR Green Master Mix and then subjected to 50 cycles of 94°C for 40s, 55°C for 40s and 72°C for 60s. Each sample was amplified in duplicate with both tryptase and β-actin primers. Negative controls of water were also included in every run. Melting curve analysis of PCR products was performed every 1°C from 50-94°C at the end of every run to ensure that a single product had been amplified.

Gene	Forward primer 5' → 3'	Reverse primer 5' → 3'
Equine tryptase	ACT GTG TTG GAC CGG ACA TT	GAC ATG GCT GGA GAT GTT GA
Equine β-actin	TGG GCC AGA AGG ACT CAT AC	CTT GAT GTC ACG CAC GAT TT

Table 5.1: Sequence of primers used in quantitative real-time PCR assays.

The crossing threshold (Ct) at which measurable fluorescence was produced was used to calculate the relative expression (RE) of tryptase transcripts to β-actin transcripts using the equation:

$$RE = \frac{2^{Ct\ \beta\text{-actin}}}{2^{Ct\ tryptase}}$$

Tryptase PCR products were also subject to Southern blotting and DIG-labelled oligo probing (section 3.3.13) to ascertain correct product amplification.

5.4. Statistical Analyses

Statistical analyses followed the format discussed in section 4.3.9. Where multiple statistical results are listed together, only the lowest non-significant value or the highest significant value is given.

5.4.1. Bronchial and Bronchiolar Mast Cell Counts

Examination of the data for the frequency of mast cells in tissues showed that neither square root nor log manipulation adequately transformed the data such that ANOVA could be performed. Confounding factors were not an issue with this data set as samples originated from one population and were treated identically. Therefore, analyses comparing the frequency of bronchial or bronchiolar mast cells in each tissue location between control and heaves horses were performed using Mann Whitney tests. Mast cell counts for bronchial and bronchiolar tissues were also combined to produce a 'total airway score', which was subject to the same inter-group comparison. If only one population (heaves or control) contained mast cells in a particular tissue location, Mann Whitney tests could not be performed. Therefore analysis was considered as the presence or absence of mast cells in the tissue and

performed using Fisher exact tests as expected counts consisted of groups of <5 (Crawley, 2002).

Data for the areas of tissue examined were also analysed by Mann Whitney tests to evaluate whether equal areas were assessed in control and heaves horses.

5.4.2. Tryptase Transcript Expression

The correction of tryptase transcript RE values for control and heaves horse BALF cell pellets for BALF mast cell number was considered. However, this normalisation was not necessary as two-tailed Wilcoxon Sign Rank tests showed no significant difference in BALF absolute mast cell count within control and heaves populations following challenge ($p=1.0$ and $p=1.0$, respectively).

Initial examination of the data showed that it could not be adequately transformed by either square root or log transformation. Mann Whitney tests were therefore used for inter-group comparisons and two-tailed Wilcoxon Sign Rank tests for intra-group comparisons.

In an attempt to verify that β-actin expression was constant in both control and heaves horses during challenge, crossing threshold values for β-actin transcripts were also compared within populations by two-tailed Wilcoxon Sign Rank tests and between populations by Mann Whitney tests.

5.5. Results

5.5.1. Bronchial and Bronchiolar Mast Cell Counts

5.5.1.1. Area of Tissues Examined

There was no significant difference between control and heaves populations in the area of epithelium, connective tissue and smooth muscle examined in tryptase stained bronchial sections ($p>0.35$). Likewise there was no significant difference between control and heaves populations in the area of epithelium, connective tissue,

smooth muscle and alveolar tissue in bronchiolar sections ($p>0.28$). Comparable areas of tissue examined were therefore analysed in control and heaves horses.

5.5.1.2. Toluidine Blue Staining

Positive cells were stained dark blue. Intra-epithelial mast cells could not be counted accurately on toluidine stained sections due to positive staining of mucus secreting cells which could not be accurately differentiated from mast cells (figs. 5.1, 5.2).

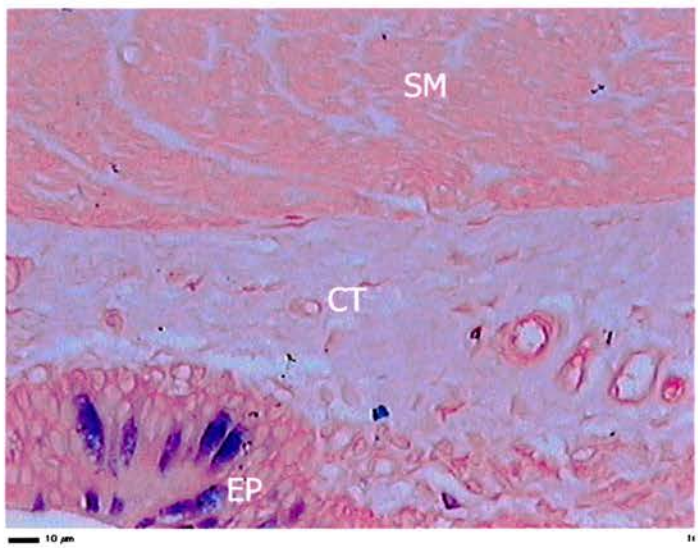

Fig. 5.1: Toluidine blue positive cells in bronchial tissue. Positive cells in the epithelium (EP) of this section look like mucus secreting cells. No positive cells are present in the smooth muscle (SM). CT = connective tissue. Size marker = 10µm.

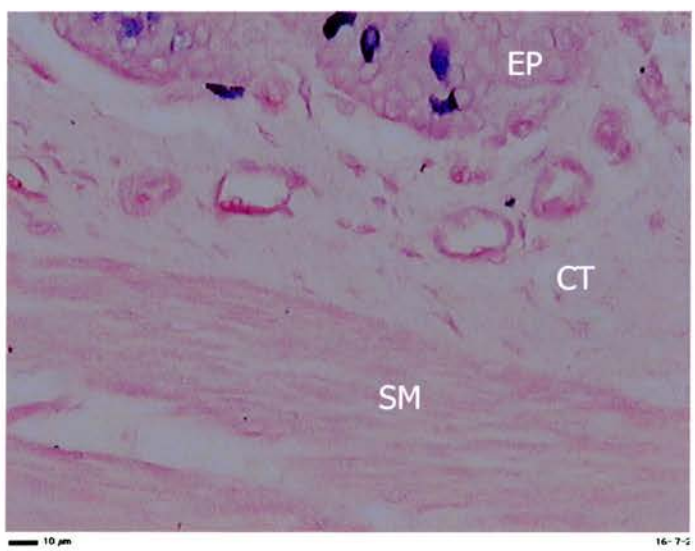

Fig. 5.2: Toluidine blue positive cells in bronchial tissue. Positive staining cells in the epithelium (EP) could not be accurately identified as mucus secreting cells or mast cells. CT = connective tissue, SM = smooth muscle. Size marker = 10µm.

The frequency of toluidine blue stained mast cells observed in bronchial and bronchiolar tissue is shown in table 5.2.

	BRONCHIAL				**BRONCHIOLAR**					
	Control		***Heaves***		***Control***			***Heaves***		
Horse	**CT**	**SM**	**CT**	**SM**	**CT**	**SM**	**ALV**	**CT**	**SM**	**ALV**
1	113.3	15.5	86.1	17.8	136.9	0	50.5	139.7	11.9	47.8
2	21.5	0	78.6	9.1	232.8	36.2	101.4	206.0	0	63.8
3	51.3	3.8	85.5	2.9	133.7	12.1	80.4	136.3	0	46.5
4	18.9	0	76.7	0	70.4	0	17.0	158.0	9.7	54.8
5	5.3	0	12.8	0	57.9	13.8	4.2	54.3	0	25.9
6	84	3.3	109.7	16.5	147.3	8.7	66.8	167.2	0	64.5
7	N/A	N/A	33.8	5.6	N/A	N/A	N/A	37.7	19.5	8.0
Median	**36.4**	**1.7**	**78.6**	**5.6**	**135.3**	**10.4**	**58.7**	**139.7**	**0**	**47.8**
Range	5.3-113.3	0-15.5	12.8-109.7	0-17.8	57.9-232.8	0-36.2	4.2-101.4	37.7-206.0	0-19.5	8.0-64.5

Table 5.2: Frequency (cells/mm^2) of toluidine blue stained mast cells in connective tissue (CT), smooth muscle (SM) and alveolar tissue (ALV) from bronchial and bronchiolar sections from control (n=6) and heaves (n=7) horses. N/A = not applicable.

There was no significant difference between control and heaves populations in the number of toluidine blue positive mast cells in connective tissue or smooth muscle of bronchial ($p>0.34$) or bronchiolar ($p>0.41$) samples. There was also no significant difference in the number of alveolar mast cells in control or heaves horses ($p=0.52$).

5.5.1.3. Tryptase Staining

Tryptase positive cells stained intensely brown throughout the entire cytoplasm (figs. 5.3, 5.4). There was no positive staining of control sections (fig. 5.5).

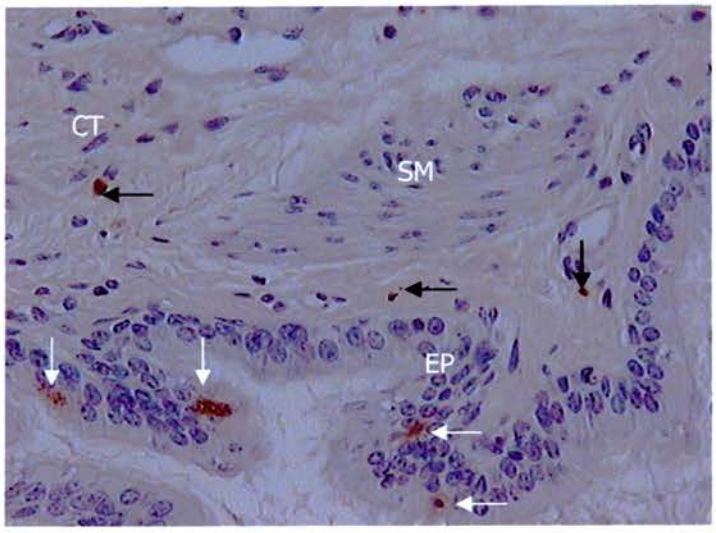

Fig. 5.3: Bronchial tissue section from a heaves horse stained with rabbit anti-equine tryptase. Tryptase positive cells are present in the epithelium (EP) (white arrows) and connective tissue (CT) (black arrows) but not the smooth muscle (SM). Size marker = 10µm.

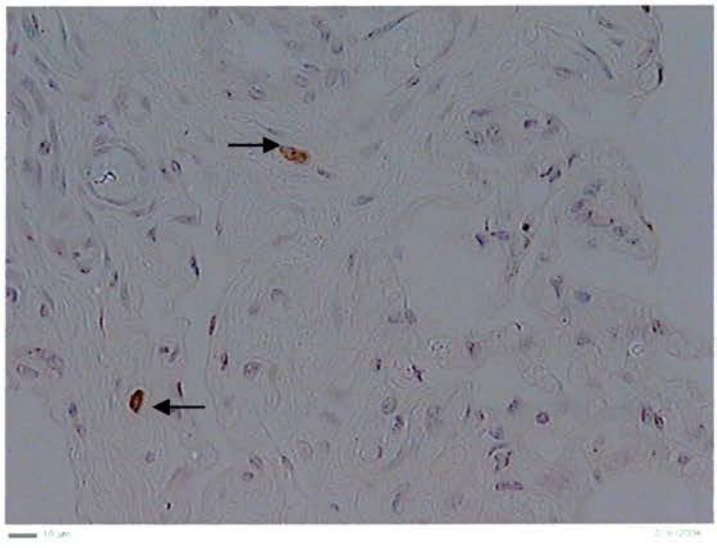

Fig. 5.4: Tryptase positive mast cells (black arrows) in alveolar tissue from a control horse stained with rabbit anti-equine tryptase. Size marker = 10µm.

Fig. 5.5: Control bronchiolar section stained with goat anti-rabbit IgG. No positive staining was observed in the epithelium (EP), connective tissue (CT) or smooth muscle (SM). Size marker = 10µm.

The frequencies of tryptase positive mast cells in bronchial and bronchiolar tissues are shown in tables 5.3 and 5.4, respectively. The total number of mast cells observed in the bronchi and bronchioles and the percentage of mast cells in each tissue location are also included for tryptase positive cells as these were the only abundant mast cell phenotype identified in all tissue locations.

	Control			***Heaves***		
Horse	**EP**	**CT**	**SM**	**EP**	**CT**	**SM**
1	0	25	4.4	9.9	2.9	5
2	0	3	0	15	37.9	0
3	0	2.4	0	10.3	9.3	5.5
4	5.5	9.6	3.2	12	47.7	10
5	89.9	86.7	18.0	0	20.6	0
6	0	0	3.8	24.5	104.6	40
7	N/A	N/A	N/A	0	18.5	3.9
Median	**0**	**6.3**	**3.5**	**10.3**	**20.6**	**5**
Range	0-89.9	0-86.7	0-18.0	0-24.5	2.9-104.6	0-40
%	0	64	36	29	57	14
Total	11.1			25.1		

Table 5.3: Frequency (cells/mm^2) of tryptase positive mast cells within bronchial epithelium (EP), connective tissue (CT) and smooth muscle (SM) in control and heaves horses. % = median number of mast cells in tissue location as a percentage of total mast cells counted in all tissue areas. Total = total number of mast cells (calculated as the median of individual horse total mast cell counts). N/A = not applicable.

	Control				*Heaves*			
Horse	**EP**	**CT**	**SM**	**ALV**	**EP**	**CT**	**SM**	**ALV**
1	0	31.9	0	29.8	4. 7	22.6	0	12.7
2	0	19.2	0	1.7	5.3	43.8	12.6	22.1
3	0	22.6	0	24.0	0	17.3	0	36.4
4	0	12.6	0	11.1	72.4	89.7	35.7	81.8
5	10.7	126.9	0	30.1	0	18.0	0	5.0
6	0	47.7	12.9	50.7	0	47.6	0	21.0
7	N/A	N/A	N/A	N/A	5.0	7.9	0	13.5
Median	**0**	**27.2**	**0**	**26.9**	**4.9**	**22.6**	**0**	**21.0**
Range	0-10.7	12.6-126.9	0-12.9	1.7-50.7	0-72.4	7.9-89.7	0-35.7	5.0-81.8
%	0	50	0	50	10	47	0	43
Total	54.2				53.7			

Table 5.4: Frequency (cells/mm^2) of tryptase positive mast cells within bronchiolar epithelium (EP), connective tissue (CT), smooth muscle (SM) and alveolar tissue (ALV) from control and heaves horses. % = mast cells in tissue location as a percentage of total mast cells and Total = total number of mast cells (calculated as the median of individual horse total mast cell counts). N/A = not applicable.

There was no significant inter-group difference in the number of tryptase positive mast cells in connective tissue or smooth muscle in bronchial ($p>0.28$) or bronchiolar ($p>0.72$) sections. Likewise, there was no significant difference in the number of tryptase positive alveolar mast cells ($p=0.94$) between control and heaves horses.

There was considerable inter-horse variation in mast cell numbers. Indeed, one control horse (control horse 5) was a statistical outlier with regard to the number of both bronchiolar and bronchial tryptase positive intra-epithelial mast cells. This was the only control horse in which bronchiolar tryptase positive intra-epithelial mast cells were identified. Additionally, while bronchial intra-epithelial mast cells were observed in one other horse (control horse 4), these were only in very low number. There was no significant difference between control and heaves horses in the number of intra-epithelial mast cells in bronchial ($p=0.28$) or bronchiolar ($p=0.32$) sections. However, a trend for increased airway epithelial mast cells in heaves horses ($p=0.09$) was evident in the total airway score. When the control outlier (control horse 5) was excluded from these comparisons, there were strong trends for increased tryptase

positive mast cells in bronchial (p=0.06) (fig. 5.6) and bronchiolar (Fisher exact test p=0.07) (fig. 5.7) epithelium. Furthermore, there were significantly increased total airway epithelial mast cells in heaves horses compared to controls (p=0.02) (fig. 5.8).

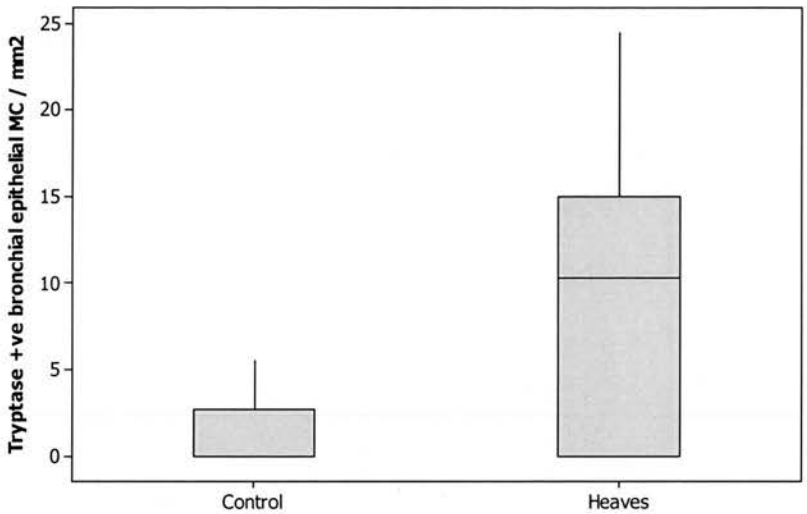

Fig. 5.6: Frequency (cells/mm^2) of tryptase positive bronchial epithelial mast cells in controls excluding horse 5 (n=5) and heaves (n=7) horses. There was a strong trend towards increased mast cells in heaves horses (p=0.06).

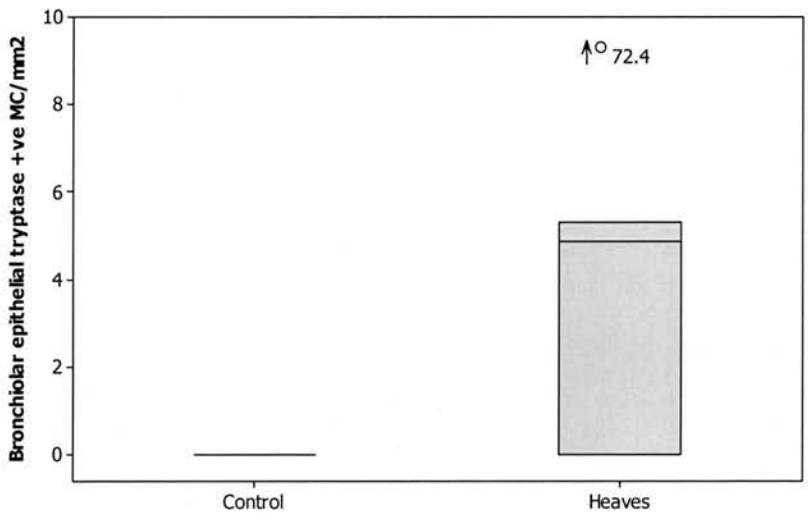

Fig. 5.7: Frequency (cells/mm^2) of tryptase positive bronchiolar epithelial mast cells in controls excluding horse 5 (n=5) and heaves (n=7) horses. There was a strong trend towards increased mast cells in heaves horses (p=0.07). ↑° denotes outlier off axis scale.

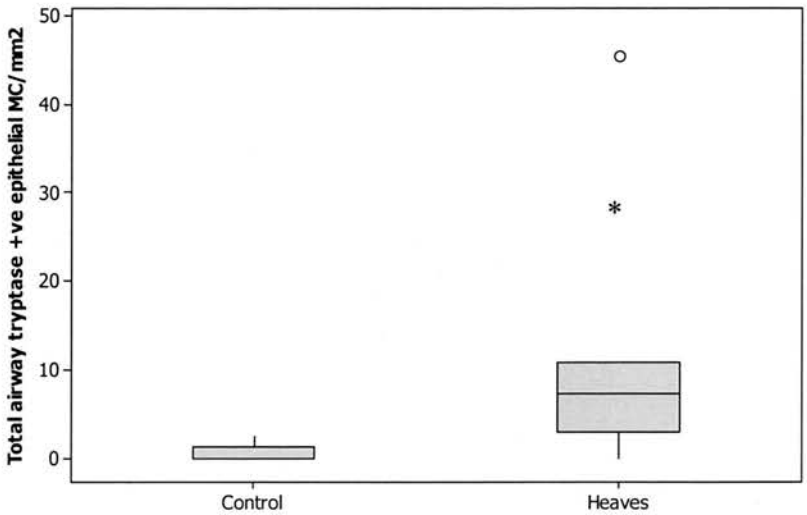

Fig. 5.8: Number of tryptase positive airway epithelial mast cells/mm^2 in controls excluding horse 5 (n=5) and heaves (n=7) horses. There were significantly increased intra-epithelial tryptase positive mast cells in heaves horses (p=0.02). * denotes significantly different to control, ° denotes outlier.

5.5.1.4. Eq.MCP-1 Staining

Eq.MCP-1 positive cells stained deep brown throughout the entire cytoplasm. There was no positive staining of control sections. The numbers of eq.MCP-1 positive mast cells/mm^2 of bronchial and bronchiolar tissues are shown in tables 5.5 and 5.6, respectively. Very few eq.MCP-1 positive mast cells were observed in the airways of either control or heaves horses. No eq.MCP-1 positive cells were observed in airway smooth muscle in either control or heaves horses. Additionally, eq.MCP-1 positive intra-epithelial mast cells were only seen in bronchial sections from two control horses.

	Control			*Heaves*		
Horse	**EP**	**CT**	**SM**	**EP**	**CT**	**SM**
1	31.4	14.9	0	0	0	0
2	0	0	0	0	0	0
3	0	12.2	0	0	0	0
4	0	0	0	0	38.3	0
5	0	0	0	0	0	0
6	4.3	0	0	0	0	0
7	N/A	N/A	N/A	0	0	0
Median	**0**	**0**	**0**	**0**	**0**	**0**
Range	0-31.4	0-14.9	0-0	0-0	0-38.3	0-0

Table 5.5: Frequency (cells/mm^2) of eq.MCP-1 positive mast cells in bronchial epithelium (EP), connective tissue (CT) and smooth muscle (SM) from control and heaves horses. N/A = not applicable.

	Control				*Heaves*			
Horse	**EP**	**CT**	**SM**	**ALV**	**EP**	**CT**	**SM**	**ALV**
1	0	12.7	0	1.8	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	2.3	0	3.1	0	0	0	0
4	0	0	0	0	0	30.7	0	38.6
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	N/A	N/A	N/A	N/A	0	0	0	0
Median	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Range	0-0	0-12.7	0-0	0-3.1	0-0	0-30.7	0-0	0-38.6

Table 5.6: Frequency (cells/mm^2) of eq.MCP-1 positive mast cells in bronchiolar epithelium (EP), connective tissue (CT), smooth muscle (SM) and alveolar tissue (ALV) from control and heaves horses. N/A = not applicable.

There was no significant difference between control and heaves horses in the number of eq.MCP-1 positive mast cells in any tissue ($p>0.50$). The presence of eq.MCP-1 was not significantly associated with control or heaves status (Fisher exact test $p=0.27$).

5.5.2. Tryptase Transcripts in BALF Cell Pellets

There was no significant change in tryptase transcript regulation in either control (p=0.4) (fig. 5.9) or heaves susceptible horses (p=0.8) (fig. 5.10) following 48h hay / straw challenge.

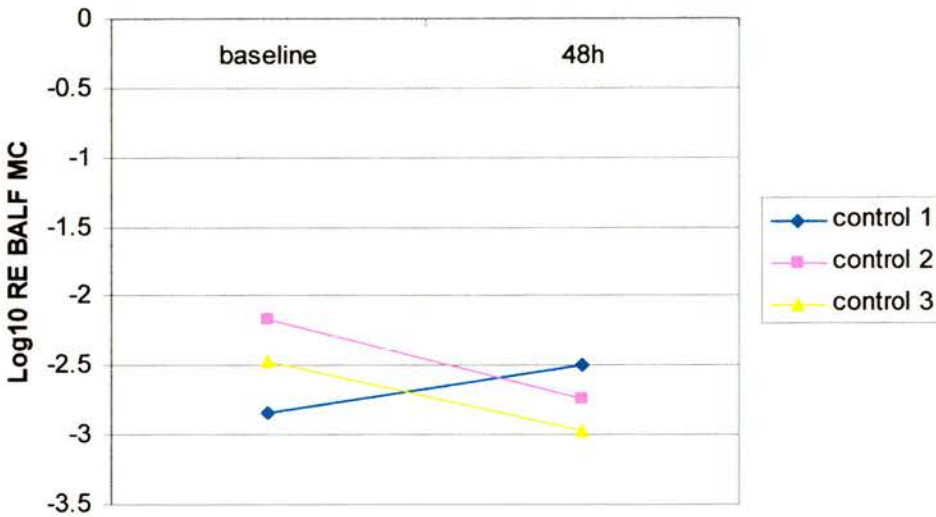

Fig. 5.9: Relative expression (RE) of tryptase transcripts compared to β-actin in control horses (n=3) pre and post 48h hay / straw challenge. There was no significant upregulation of tryptase transcripts during challenge (p=0.4).

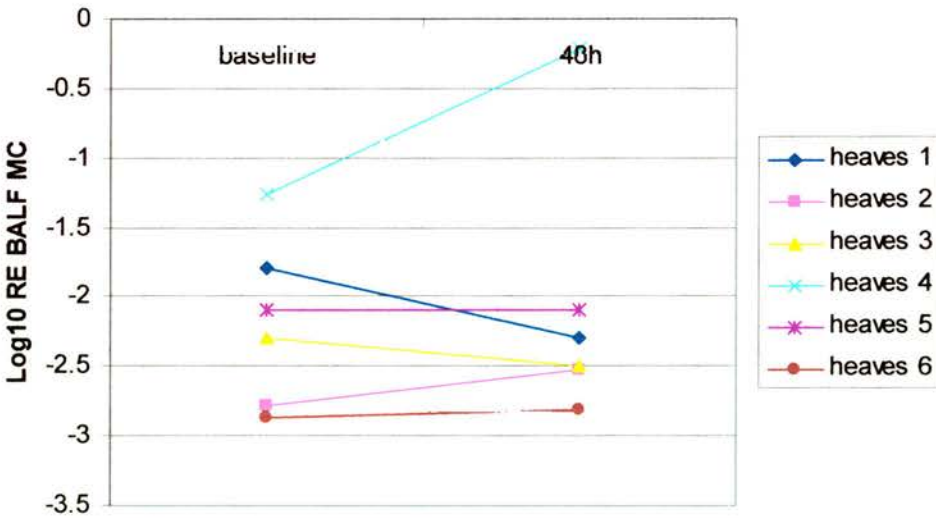

Fig. 5.10: Relative expression (RE) of tryptase transcripts compared to β-actin in heaves susceptible horses (n=6) pre and post 48h hay / straw challenge. There was no significant upregulation of tryptase transcripts during challenge (p=0.8).

β-actin transcripts were not significantly different in control (p=1.0) or heaves (p=0.5) horses following challenge. Similarly, there was no significant difference in β-actin transcripts between control and heaves horses at baseline (p=0.2) or at 48h (p=0.2).

5.5.3. Tryptase Transcripts in Bronchial and Bronchiolar Tissue

There was no significant difference in bronchial tryptase transcripts between controls and heaves horses in the early resolution phase (p=0.7) (fig. 5.12). However, bronchiolar tryptase transcripts were down regulated seven-fold in early resolution heaves horses compared to controls (p=0.02) (fig. 5.13).

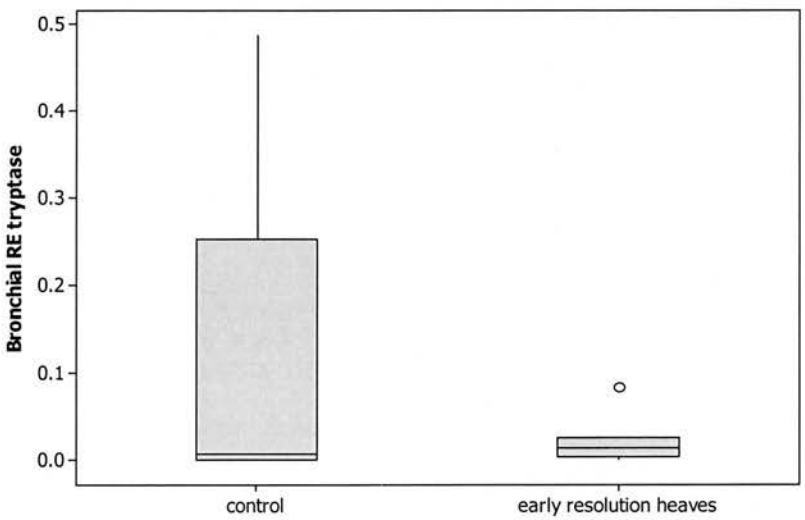

Fig. 5.12: Relative expression (RE) of tryptase transcripts compared to β-actin in bronchial tissue from control (n=6) and heaves horses (n=7) in the early resolution phase following hay / straw challenge. There was no significant difference in tryptase transcripts between the two populations (p=0.7), ° denotes outlier.

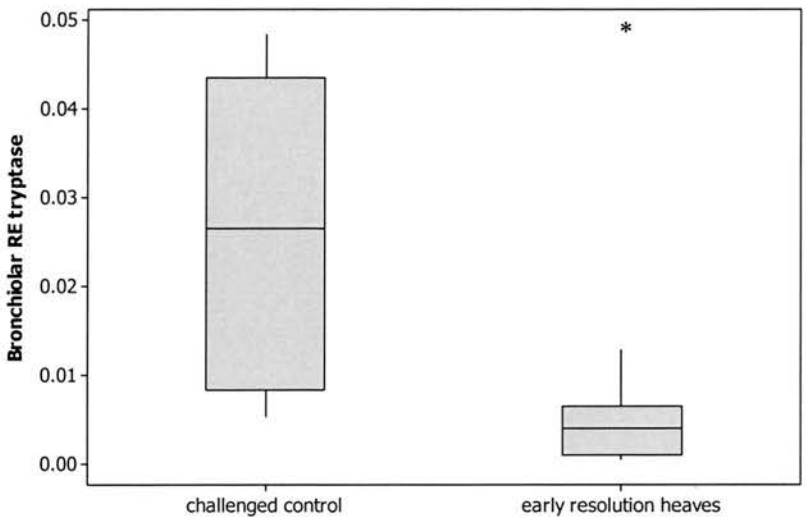

Fig. 5.13: Relative expression (RE) of tryptase transcripts compared to β-actin in bronchiolar tissue from control (n=6) and heaves horses (n=7) in the early resolution phase following hay / straw challenge. Tryptase transcripts in heaves horses were down-regulated seven-fold compared to controls (p=0.02), * denotes significantly different to challenged control.

There was no significant difference in β-actin transcripts between control and early resolution heaves horses in bronchial (p=0.2) or bronchiolar tissues (p=0.4).

5.6. Discussion

5.6.1. Bronchial and Bronchiolar Mast Cell Populations

5.6.1.1. General Mast Cell Population Characteristics

Tryptase positive mast cells were abundant while eq.MCP-1 positive mast cells were scarcely observed in the equine lung, as previously noted by Pemberton *et al.* (2001), confirming that equine pulmonary mast cells are predominantly tryptase positive, chymase negative in their proteinase content. This is not surprising since it is well documented across species that the lung contains predominantly tryptase positive, chymase negative mast cells (Bienenstock *et al.,* 1985; Irani *et al.,* 1986; Schwartz *et al.,* 1987; Wenzel *et al.,* 1988; Chen *et al.,* 1993; Lavens *et al.,* 1993; Gibson *et al.,* 1993; Kuther *et al.,* 1998; Jolly *et al.,* 1999). Intra-epithelial mast cells, in particular,

were almost exclusively of this phenotype, again as reported in other species (Bienenstock *et al.,* 1985; Irani *et al.,* 1986). Interestingly, the two horses (both controls) with eq.MCP-1 positive bronchial intra-epithelial mast cells were different control horses from those with tryptase positive bronchial intra-epithelial mast cells. This suggests that a population of chymase positive, tryptase negative mast cells may exist in the equine lung, as has been reported in cattle and dogs (Kube *et al.,* 1998; Kuther *et al.,* 1998; Jolly *et al.,* 1999). Indeed, occasional single eq.MCP-1 labelled mast cells were observed on dual tryptase and eq.MCP-1 labelled equine lung sections (section 2.5.3.3).

There is some disparity between numbers of tryptase positive and toluidine blue positive mast cells counted in the airways. As discussed, effectively all equine pulmonary mast cells are tryptase positive and therefore tryptase and toluidine blue positive mast cell numbers should be closely correlated. However, equine basophils are also reported to stain with toluidine blue (Dirscherl *et al.*, 1993) as are canine and simian basophils (Patterson *et al.*, 1974). It therefore seems likely that basophils may contribute to the toluidine blue stained cell counts in this study. Although basophils are traditionally thought to be confined to the peripheral bloodstream, they have been observed in tissue locations, including human airway tissues (Okuda *et al.*, 1983; Koshino *et al.*, 1995). Although Koshino *et al.* (1995) reported increased numbers of basophils in asthmatic airways compared to controls, the number of toluidine blue positive cells (presumed to include basophils) is not elevated in heaves horses compared to controls. Due to this potential exaggeration of the toluidine blue mast cell count by basophils, tryptase positive cell counts will represent mast cell enumeration in the tissues more accurately. Consequently, tryptase positive mast cell values have been used for comparison with other studies.

The majority of toluidine blue and tryptase positive mast cells in the equine lung were located in the alveoli and connective tissue with a smaller population resident in the epithelial layer. This finding is consistent with a previous equine study (Mair *et al.*, 1988) and concurs with studies in other species (Craig *et al.,* 1986; Shanahan *et al.,* 1987; Chen *et al.,* 1990; Kuther *et al.,* 1998). Likewise, there was no significant

difference in alveolar and connective tissue mast cell numbers between control and heaves horses, similar to studies comparing control and asthmatic humans (Djukanovic *et al.,* 1990; Pesci *et al.,* 1993; Laitinen *et al.,* 1993; Ammit *et al.,* 1997).

In contrast to the alveoli and connective tissue, very few mast cells were observed in the smooth muscle of either control or heaves horses, as has also been reported in normal canine lung tissue (Kube *et al.*, 1998). High numbers of smooth muscle mast cells are reported in the human lung (Carroll *et al.,* 2002a) with further recruitment in asthmatics (Ammit *et al.*, 1997; Carroll *et al.*, 2002a) where tryptase is believed to act as both a direct and indirect agonist and mitogen of smooth muscle (Chambers *et al.,* 2001; Berger *et al.,* 2001a; Berger *et al.,* 2001b; Brown *et al.,* 2002; Cho *et al.,* 2003). These results suggest that mast cell hyperplasia is not a pre-requisite for the pulmonary smooth muscle hyperplasia observed in heaves (Ramos-Barbon *et al.*, 2004). This was somewhat surprising as mast cell mediators have been shown to augment cholinergic airway tone in the equine airway and have been suggested as an important mechanism in the development of airway obstruction in heaves (Olszewski *et al.*, 1999). It is still possible however that mast cell mediators diffuse from surrounding tissue to act on smooth muscle in heaves horses.

It is difficult to directly compare the observed frequency of tissue mast cells to other studies evaluating mast cell populations in horses and other species due to inconsistencies in study design. These include differences in tissue fixation, staining and method of mast cell enumeration and consequently reported figures are highly variable. As pulmonary mast cells are predominantly of the mucosal mast cell type, studies using formalin fixation, which interferes with staining of these cells, are likely to underestimate mast cell numbers. Electron microscopy studies report the highest values, presumably due to this technique allowing recognition and enumeration of degranulated or 'ghost' mast cells which are likely be missed by mast cell staining techniques that rely on staining granule constituents.

Winder and von Fellenberg (1990) and Mair *et al.* (1988) have previously reported mast cell populations of the equine lung using similar methodology to this current study (Carnoys fixed tissue and toluidine blue staining). Whilst mast cell numbers in the current study are similar to those reported in the former paper, they are lower than those described by Mair *et al.* (1988). However the latter study included intra-epithelial mast cells and may possibly have over-estimated mast cell numbers due to the difficulty in distinguishing these intra-epithelial mast cells from mucus secreting cells, as reported in this study and by other authors (Nicholls, 1978; Winder and von Fellenberg, 1990).

There was an increased number of mast cells in bronchiolar compared to bronchial tissues, a trend which has previously been reported in horses (Nicholls, 1978) and other species (Guerzon *et al.,* 1979; Lamb and Lumsden, 1982; Chen *et al.,* 1990; Carroll *et al.,* 2002a). The majority of airway mast cells were located in the connective tissue compartment, as previously reported in healthy horses (Mair *et al.*, 1988) and other species (Guerzon *et al.*, 1979; Shanahan *et al.*, 1987; Kube *et al.*, 1998; Jolly *et al.*, 2000). Mair *et al.* (1988) reported that the majority of mast cells associated with the airways of healthy horses resided subepithelially within the lamina propria connective tissue, a pertinent location for recruitment to the epithelium during challenge in heaves susceptible individuals. In the current study, mast cells in the lamina propria were not counted as a distinct population but were included in the connective tissue count and therefore we are unable to comment on subepithelial mast cell populations.

Increased (total) numbers of bronchiolar mast cells are reported in horses suffering from bronchiolitis compared to controls (Winder and von Fellenberg, 1990) suggesting recruitment of mast cells to the bronchioles during an inflammatory response. This was not observed in the current study however the aetiology(ies) of bronchiolitis in the study by Winder *et al.* (1990) was not disclosed and therefore may have included other causes of bronchiolitis in addition to heaves such as neoplastic, fibrotic and interstitial diseases, which can lead to elevated mast cell counts in humans (Walls *et al.*, 1990).

Specific staining characteristics and comparisons with other studies are further discussed below. Many studies have been performed in individuals with defined type I mast cell mediated allergies such as allergic rhinitis. Caution should be exercised in comparing heaves with these allergic conditions as the role of allergy in heaves has not been definitively confirmed. Furthermore, the pathophysiological consequences of non-immunological mast cell activation may differ from allergy driven mast cell activation. Therefore, studies confined to investigating mast cell involvement in type I allergic diseases have only been used for comparison with this study when data in other diseases is lacking.

5.6.1.2. Toluidine Blue Staining

As positive staining of mucus secreting cells was observed with toluidine blue, it was not possible to accurately evaluate epithelial mast cells with this stain. This may be a peculiarity specific to the horse as only two other reports, also in the horse, have noted this effect (Nicholls, 1978; Winder and von Fellenberg, 1990), which is presumed to be a result of toluidine blue staining sulphated mucopolysaccharides (Culling, 1963) which both equine mucus and mast cells contain (Nicholls, 1978).

5.6.1.3. Tryptase Staining

Using the total airway score, a trend for increased tryptase positive intra-epithelial mast cells was present in heaves horses compared to controls; however, this data was skewed by the outlier control horse 5. This outlier control had been owned by Michigan State University for many years and had undergone rigorous testing to ensure control status. However, other diseases which can elevate pulmonary mast cell counts as previously discussed may have been present but undetected. Unfortunately, due to unidentified problems, lung samples from this horse disintegrated in RNA later and consequently could not be used for quantitative RT-PCR which may have yielded further information. Subsequent examination of lung tissue from this horse by a pathologist did not reveal any histopathological abnormalities. When this outlier horse was excluded, there were significantly increased total airway intra-epithelial mast cells in heaves horses compared to

controls. Likewise, when control horse 5 was excluded, trends for increased intra-epithelial mast cells in heaves horses also became evident in bronchial and bronchiolar data subsets. As the number of horses used in this study was low, it is possible that some non-significant results are due to lack of statistical power resulting from small sample sizes.

The intra-epithelial accumulation of mast cells in heaves horses noted in this study is in agreement with the ultrastructural study performed by Kaup *et al.* (1990b), which showed many of these mast cells to be degranulated. Except for the outlier horse, intra-epithelial mast cells were rarely observed in controls. Similarly, intra-epithelial mast cells are reportedly rare in control human subjects but are increased in the lungs of human asthmatics (Lozewicz *et al.,* 1988; Laitinen and Laitinen, 1988; Crimi *et al.,* 1991; Di Stefano *et al.,* 1993; Pesci *et al.,* 1993; Gibson *et al.,* 1993; Laitinen *et al.,* 1993). Increased intra-epithelial mast cells are also reported in the lungs of chronic bronchitis patients (Pesci *et al.*, 1994) providing evidence for epithelial mast cell recruitment in non-allergic pulmonary disease.

The number of intra-epithelial mast cells in remission heaves horses could not be evaluated as tissues were not available from these horses. Asymptomatic, pre-seasonal allergic rhinitis sufferers have reduced numbers of intra-epithelial nasal mast cells compared to when symptomatic, but greater numbers than control subjects, suggestive of some degree of persistent immunological activation of the airway mucosa (Viegas *et al.,* 1987; Juliusson *et al.,* 1995). Following challenge of asymptomatic allergic rhinitis sufferers, mast cell migration into the nasal epithelium has been observed (Kawabori *et al.,* 1985; Viegas *et al.,* 1987). Indeed, in a canine antigen challenge model, airway intra-epithelial mast cells increased in the lung as rapidly as one hour following challenge (Turner *et al.*, 1988). Treatment with the corticosteroid beclomethasone propionate inhibits the seasonal increase in nasal mucosal mast cells in allergic rhinitis sufferers (Gomez *et al.*, 1988) and reduces the numbers of intra-epithelial mast cells in symptomatic allergic rhinitis patients (Otsuka *et al.*, 1986; Juliusson *et al.*, 1995). These observations entice speculation

that a similar mechanism may be involved in the clinical response of heaves affected horses to corticosteroid administration.

Concurring with the current study, intra-epithelial mast cells in the airway have repeatedly been shown to be almost exclusively tryptase positive in proteinase content in humans (Bentley *et al.,* 1992; Gibson *et al.,* 1993; Aldenborg and Enerback, 1994; Juliusson *et al.,* 1995). This epithelial accumulation of mast cells in both allergic and non-allergic asthma and also diseases unrelated to allergy such as chronic bronchitis, indicate that this recruitment can be a feature of airway inflammation *per se* rather than being specific to allergy. Although only a low proportion of total mast cells are in an intra-epithelial location, comprising just 0-2% of epithelial cells (Lamb and Lumsden, 1982), it is suggested that this is a substantial number of cells when the whole lung surface is considered (Guerzon *et al.*, 1979). Positive correlations have been observed between the number of intra-epithelial mast cells in bronchial brushings and airway hyperreactivity and airway obstruction in human asthmatics (Gibson *et al.*, 1993). Furthermore, electron microscopic evidence of many partially or even fully degranulated mast cells in the pulmonary airway mucosa has been documented in numerous studies (Fox *et al.,* 1981; Lamb and Lumsden, 1982; Claman *et al.,* 1986; Laitinen and Laitinen, 1988; Chen *et al.,* 1990; Djukanovic *et al.,* 1990; Heard *et al.,* 1990; Di Stefano *et al.,* 1993; Pesci *et al.,* 1993; Laitinen *et al.,* 1993; Beil and Pammer, 2001; Carroll *et al.,* 2002a). These degranulating and degranulated mast cells appear to be activated, possibly in an attempt to replenish their stores of granule contents (Claman *et al.*, 1986). Some mast cell degranulation is reported to occur in airways from control individuals (Fox *et al.,* 1981; Lamb and Lumsden, 1982) and a gradient of mast cell degranulation has been identified in normal human bronchi which appears to increase in magnitude from the deep submucosa to superficial mucosa and epithelium (Heard *et al.*, 1990). However, increased numbers of degranulating and degranulated intra-epithelial mast cells are observed in asthmatics (Beasley *et al.,* 1989; Djukanovic *et al.,* 1990; Di Stefano *et al.,* 1993; Pesci *et al.,* 1993; Carroll *et al.,* 2002a). Similarly, airway epithelium from heaves horses is reported to contain intra-epithelial accumulations of mast cells, the majority of which were in varying stages of degranulation (Kaup *et al.,* 1990b). Mast

cell degranulation can not be reliably assessed on stained sections and as electron microscopy was not performed as part of the current study, we are unable to comment on levels of mast cell degranulation in equine airway epithelium. However, if total degranulation of mast cells occurs in the airway of heaves horses as has been described in asthmatic airways, the number of intra-epithelial mast cells counted by immunohistochemistry is likely to have been underestimated.

In the current study, the increase in intra-epithelial mast cells without increased total bronchiolar mast cells suggests re-distribution of resident bronchiolar mast cells with migration to the epithelium, rather than recruitment of new cells. This concurs with a previous canine allergic model in which early pulmonary epithelial mast cell recruitment was followed by decreased mast cell counts at 10h post challenge (Turner *et al.*, 1988). This was attributed to mast cell degranulation and the inability to visualise these 'ghost cells', rather than movement of mast cells back out of the epithelium as mast cells were not increased in the subepithelial area or airway lumen (Turner *et al.*, 1988). It is suspected, but not conclusively proven, in human allergic airway disease that intra-epithelial mast cells interact with inhaled allergens and degranulate into the airway contributing to elevated histamine and proteinase concentrations in BALF. Considering the number of intra-epithelial mast cells is increased in heaves horses, but the number of luminal mast cells is not, it seems likely that these intra-epithelial mast cells also contribute significantly to the increased BALF tryptase concentration measured in these horses (Chapter 4).

Gibson *et al.* (1993) found a positive correlation between the number of mast cells in the epithelium and in the BALF in asthmatics, although intra-epithelial mast cells outnumbered luminal mast cells by a factor of ten. BAL may wash mast cells from the epithelial surface and / or mast cells may be extruded through the epithelium into the airway lumen, the latter potentially being a clearing process for senescent cells. Another clearance mechanism could be mast cell apoptosis and engulfment by phagocytic cells and certainly phagocytosed mast cells in macrophages have been observed in electron microscopy studies of the human airway (Walls *et al.,* 1990; Beil and Pammer, 2001). Tryptase positive macrophages and neutrophils observed in

immunolabelled equine BALF cytospins (Chapter 4) was also suggestive of mast cell phagocytosis occurring in the equine airway lumen.

5.6.1.4. Eq.MCP-1 Staining

Eq.MCP-1 positive cells were much less frequently observed in airway tissues than tryptase positive cells concurring with reports in other species that pulmonary mast cells are predominantly tryptase positive, chymase negative in proteinase content (Schwartz *et al.,* 1981; Irani *et al.,* 1986; Wenzel *et al.,* 1988; Lavens *et al.,* 1993; Gibson *et al.,* 1993). There was no significant inter-group difference in the number of eq.MCP-1 positive cells in any tissue. Chymase positive mast cells are reported to decrease in inflammatory conditions associated with mast cell activation and degranulation (Walls *et al.*, 1990). It therefore might be expected that fewer eq.MCP-1 positive cells would be observed in heaves horses. However, in this study, the presence of eq.MCP-1 stained cells was not significantly associated with control or heaves status.

Other than rodents, which appear to have different mast cell phenotypes to most other species, chymase positive mast cells are found in greatest proportions in connective tissue compartments and are largely absent from epithelium (Jolly *et al.*, 2000) as observed in this study. Interestingly, a pilot study for immunohistochemical staining techniques found that eq.MCP-1 positive cells were abundant in intestinal mucosa and therefore may have more similarity to rodent chymases.

In human bronchi, numbers of chymase positive mast cells are reported to be highest around submucosal glands (Matin *et al.*, 1992) which, given the potent secretagogue properties of chymase (Sommerhoff *et al.,* 1989a), would seem a pertinent microenvironment. In the current study, peri-glandular mast cells were not counted as a distinct population and were incorporated into the connective tissue count.

Beil and Pammer (2001) demonstrated high numbers of dual tryptase and chymase positive mast cells in human lung parenchyma and suggested that prior studies had not used optimal tissue processing (reportedly 4.5% formalin, for 6h, followed by

70% ethanol until processing). All tissues used in the current study were collected immediately post mortem into Carnoys fixative. Preliminary trials had shown preferential staining of Carnoys fixed tissue compared to very poor staining, particularly of mucosal mast cells, with formalin fixed equine tissues as previously reported (Nicholls, 1978; Winder and von Fellenberg, 1990). Species variation in optimal mast cell fixation and staining techniques, as well as disparity in mast cell proteinase content with anatomical site and disease status is well recognised (Bienenstock *et al.*, 1985; Shanahan *et al.*, 1987; Walls *et al.*, 1990; Kube *et al.*, 1998). These factors may well explain the inconsistencies between studies and the lower proportion of chymase positive mast cells observed in the equine lung. Furthermore, as eq.MCP-1 is a β-chymase, its distribution may not be comparable with human chymase which is an α-chymase. It is also possible that tissues used by Beil and Pammer (2001) had some upregulation of chymase as has been reported, for example, in fibrotic lung disease and pulmonary hypertension (Walls *et al.,* 1990; Hamada *et al.,* 1999) as fibrotic alveolar septae were reported to be commonly observed.

Sture *et al.* (1995) also report higher numbers of chymase positive cells in the sheep lung than the current study with a mean ± SD of 23.8 ± 4.3 and 51.3 ± 18.3 sheep mast cell proteinase positive cells/mm^2 in ovine bronchial and lung tissue respectively. Sheep mast cell proteinases have since been further classified into a number of subtypes (McAleese *et al.*, 1998) and sheep mast cell proteinase-1 shown to be a dual specific proteinase with both tryptase and chymase properties (Pemberton *et al.*, 1997). Therefore the distribution reported by Sture *et al.* (1995) may not reflect that of a mono-specific chymase.

5.6.2. Quantitative RT-PCR and Tryptase Transcript Expression

5.6.2.1. BALF Cell Pellets

It was hypothesised that luminal mast cells from heaves susceptible horses might upregulate tryptase mRNA expression following hay / straw challenge in order to increase tryptase production. This would account for the observation that BALF

tryptase concentrations are increased in heaves horses (Chapter 4) without a concurrent increase in BALF mast cell number. However, the lack of upregulation of tryptase transcripts in both control and heaves susceptible horses following 48h hay / straw challenge suggests that these luminal mast cells may not be capable of increasing tryptase expression following challenge. It is feasible, although considered unlikely, that upregulation was missed by an inappropriate time-point. Therefore it appears that luminal mast cells in heaves susceptible horses can contribute to the increased BALF tryptase concentration only by degranulation and can not respond to challenge by upregulation of tryptase expression. Increased histamine releasability of airway luminal mast cells following *in vitro* allergen challenge is reported in heaves horses and allergic dogs compared to controls (Patterson *et al.,* 1974; Hare *et al.,* 1999). However, human airway luminal mast cells release significantly less histamine than dispersed parenchymal mast cells in response to calcium ionophore (Casolaro *et al.*, 1989). Therefore it seems likely that whilst luminal mast cells contribute to the increased BALF tryptase concentrations observed in heaves horses, tissue mast cells are the more potent participants in response to challenge. This hypothesis is further supported by the elevated numbers of intra-epithelial mast cells observed in heaves horses compared to controls. The inability of luminal mast cells to upregulate tryptase expression in response to challenge suggests that these cells, and possibly all luminal cells, may be an older, senescent cell population. This is further supported by other quantitative studies which have failed to detect upregulation of cytokine expression in luminal cells from challenged asthmatics (Berkman *et al.*, 2001; Muhlebach *et al.*, 2004).

5.6.2.2. Bronchial and Bronchiolar Tissues

Bronchial tissue did not show any change in tryptase transcript regulation supporting the belief that heaves is predominantly a disease of the small airways with comparatively little contribution from the larger conducting airways. Only one specific time-point was investigated however, and therefore involvement of bronchial tissue at other time-points can not be excluded.

In contrast, bronchiolar tissue from heaves horses in early resolution phase showed a significant, seven-fold decrease in tryptase transcript expression compared to control horses. This suggests that bronchiolar mast cells had 'switched off' tryptase mRNA expression following cessation of challenge. The decreased BALF tryptase concentrations of heaves horses in remission compared to clinical heaves horses (Chapter 4) suggests that this down-regulation of tryptase expression is continued through to the protein level. This down-regulation was observed despite the presence of increased numbers of intra-epithelial mast cells in these horses (section 5.5.1.3). These results suggest that bronchiolar mast cells, in contrast to luminal mast cells, are capable of altering tryptase expression in response to the challenge environment. This, and the increase in intra-epithelial tryptase positive mast cells in heaves horses post challenge invites speculation that tryptase upregulation may occur in bronchiolar mast cells during clinical exacerbation of heaves. Ideally samples would also have been collected from heaves horses during acute exacerbation of heaves to investigate this possibility.

Although there are no previous investigations of mast cell mediator regulation in the lung or in airway luminal cells, quantitative studies of cytokine expression can be used as a comparison of the response of these two compartments. As previously mentioned there are no studies confirming upregulation of proteinase or cytokine expression by airway luminal mast cells. In contrast however, cytokine upregulation has been demonstrated in mucosal mast cells in nasal biopsies from allergic rhinitis patients and in lung mast cells in an asthma model, thus supporting the hypothesis of tissue mast cell response to challenge (Jaffe *et al.,* 1995; Pawanker *et al.,* 2000). Indeed, antigen-activated nasal mast cells secreted greater levels of IL-4 and IL-13 than antigen-activated nasal T lymphocytes (Pawanker *et al.*, 2000).

It seems probable that mast cells most accessible to the airway, i.e. intra-epithelial and alveolar mast cells, would respond to challenge by alteration of mRNA expression. During challenge these cells may be activated and upregulate tryptase mRNA transcripts with subsequent increased expression at the protein level. These mast cells may then pass into the airway lumen themselves when no longer able to

upregulate mRNA expression but still able to degranulate stored tryptase. If indeed intraluminal mast cells are a senescent population, tryptase release from these cells may also be partly due to autolysis, a concept supported by the apparent reduced viability of mast cells compared to other luminal cells in allergic dogs (Patterson *et al.*, 1974). Alternatively, mast cells could be primed and upregulated in the tissues before migrating into the lumen with maximal reserves of tryptase available for degranulation. However, the lack of mast cell recruitment to the airway lumen in clinical heaves (McGorum *et al.,* 1993d; Dixon *et al.,* 1995b) and the presence of degranulating and degranulated mast cells in the airway epithelium of healthy horses (Mair *et al.*, 1988) suggests that this latter explanation is unlikely.

5.6.2.3. β-actin Expression

The valid use of house-keeping genes in quantitative mRNA assays to normalise gene-of-interest data for variations in processing and signal quantitation relies upon their constant cellular expression. House-keeping genes are defined by specific gene promoter elements which determine that they are expressed constitutively in every cell. However, this does not necessarily mean that their expression is not regulated and it is virtually impossible to find the perfect house-keeping gene whose expression is unaffected by cellular proliferation, activation or differentiation (Thellin *et al.,* 1999; Glare *et al.,* 2002).

β-actin, a cytoskeletal protein, was used in this study due to its reported use as a house-keeping gene in the equine airway (Giguere *et al.,* 2002; Bowles *et al.,* 2002) and the ability to design specific primers from the published equine cDNA sequence. Recently variable β-actin expression in asthmatic airways has been reported (Glare *et al.*, 2002) casting doubt on its validity as a house-keeping gene. β-actin expression was therefore compared between control and heaves horses in all quantitative assays performed. No significant difference in β-actin expression was found during challenge of either control or heaves horses or between the two populations of horses such that it appears to be a suitable house-keeping gene in this disease model. Although this is only a very crude measure of house-keeping gene validity, other methods of verification are very time-consuming and difficult to perform.

5.7. Conclusion

During disease exacerbation, heaves susceptible horses have increased numbers of tryptase immunoreactive intra-epithelial mast cells compared with control horses. This suggests a role for these cells in contributing to the increased BALF tryptase concentrations reported in heaves susceptible horses post challenge in Chapter 4. Furthermore, challenge of heaves horses failed to elicit upregulation of tryptase mRNA expression in airway luminal mast cells whereas down-regulation of tryptase occurred in bronchiolar mast cells following removal of challenge. Collectively, these results imply that tissue mast cells may be more important in the development and resolution of the pulmonary inflammatory response in heaves than luminal mast cells. The response of luminal mast cells to organic dust challenge is investigated further in Chapter 6.

An increase in mast cells in the smooth muscle compartment was not observed in heaves horses suggesting that they are not critical to the development of bronchospasm in these horses. This finding contrasts with observations in human asthmatics.

Chapter 6: *In Vitro* Mast Cell and Tracheal Explant Challenges

6.1. Summary

To investigate whether hay dust can induce mast cell degranulation, mixed cell pellets recovered from BALF from control (n=6) and heaves susceptible (n=6) horses in remission were challenged with hay dust suspension (HDS). Supernatant tryptase content could not be measured due to interference with the ELISA by unidentified components of the HDS, and therefore an indication of mast cell degranulation was determined by measurement of residual tryptase in lysed cell pellets. Equine airway luminal mast cells from heaves susceptible horses showed significant degranulation with 75 and 7.5mg/ml HDS challenge. A trend for dose dependent degranulation was evident in both control and heaves susceptible horses following challenge with HDS, which suggests involvement of a non-IgE mediated mast cell response to challenge. As no attempt was made to isolate a pure mast cell population, other cell types or their products, may have contributed to mast cell degranulation. The increased number of intra-epithelial mast cells in heaves horses compared to controls may be important in the *in vivo* mast cell response to HDS challenge, such that increased BALF tryptase concentrations only occur in heaves horses.

A potential secretagogue role for equine mast cell proteinases was investigated using air-liquid interface tracheal explant culture. Explants harvested from 3 control horses were challenged with tryptase, eq.MCP-1 and eq.MCP-1 plus soy bean trypsin inhibitor. Mucus quantitation, as measured by optical density (OD) of explant PBS washings incubated with Periodic Acid Schiff (PAS) reagent and by real-time RT-PCR of equine mucin gene MUC-5AC expression, was not increased following incubation with either mast cell proteinase. Therefore, equine mast cell proteinases may not act as secretagogues on mucus secreting cells. However, it was considered more likely that the high concentrations of mast cell proteinases achieved *in vivo* by

mast cell degranulation local to mucus glands may not have been achievable using explants due to poor tissue penetration or inactivation of proteinases.

6.2. Introduction

In Chapter 4 it was demonstrated that heaves susceptible horses have increased BALF tryptase concentrations following inhalational hay / straw challenge. However, the relative contribution of airway luminal and intra-epithelial mast cell degranulation to this increase in tryptase concentration is unknown. Results from Chapter 5 suggest that intra-epithelial mast cells may contribute substantially to degranulation and that airway luminal mast cells may be less important in the pulmonary inflammatory response in heaves horses. Challenge of airway luminal mast cell populations recovered from BALF would therefore confirm their ability to degranulate in response to hay dust.

Mast cell degranulation has traditionally been viewed to occur as a result of allergen specific crosslinkage of IgE to high affinity receptors (FcεRI) on the mast cell surface. However, more recently neuropeptides, complement, tryptase, endotoxin, lectins, cytokines, adenosine and changes in osmolality have also been reported as alternative means of mast cell activation and degranulation, with neuropeptide and cytokine activation, in particular, thought to be of biological significance in the asthmatic airway (Church *et al.,* 1989; Bingham and Austen, 2000; Holgate, 2000; Hart, 2001). Hay dust, like grain dust, contains a number of pro-inflammatory agents including endotoxin, β-D-glucan, serine and metalloproteinases and particulates (Pirie *et al.,* 2002b), all of which may be capable of direct mast cell activation. Grain dust has been shown to stimulate histamine release from human lung fragments in a dose-dependent manner, independent of complement, cell cytotoxicity and IgE (Chan-Yeung *et al.*, 1987). Indirect mast cell activation by grain dust has also been demonstrated via induction of a histamine releasing factor from lymphocytes (Alam *et al.*, 1988). Although increased BALF IgE has been reported in heaves horses compared to controls (Halliwell *et al.*, 1993; Schmallenbach *et al.*, 1998), a definitive role for IgE, and indeed allergy, in the pathogenesis of equine heaves has not been

established. Therefore, this chapter hopes to further elucidate the role of allergy in equine heaves by considering the response of both control and heaves susceptible horses to HDS.

This study has identified increased tryptase concentrations in BALF of horses with heaves, consistent with a potential role for mast cells in pathophysiology of heaves. However, further work is required to determine the consequences of these increased tryptase concentrations on airway inflammation and function in heaves. Tryptase release in the lung could have several potential pro-inflammatory consequences including smooth muscle contraction and hyperplasia, increased vascular permeability, neuropeptide regulation and recruitment of inflammatory cells as described in section 1.4.4.1. Trypsin is reported to be a secretagogue for goblet cells in rodent airway epithelium (Niles *et al.*, 1986). Intra-epithelial tryptase positive mast cells observed in heaves horses (Chapter 5) are therefore in an ideal microenvironment to stimulate local mucus secretion during clinical disease. Mast cell chymase is also an important secretagogue for serous cells. Indeed, it is the most potent secretagogue for cultured serous cells identified to date, with respect to threshold concentration and magnitude of response (Sommerhoff *et al.,* 1989a). Although chymase positive cells are not present in large numbers in the human airway, their strategic location around submucosal glands is considered to have physiological importance in the exaggerated mucus production of asthmatics (Matin *et al.,* 1992; Carroll *et al.,* 2002b). A similar role in equine heaves may contribute to the mucus hypersecretion associated with clinical exacerbation of disease. Potential secretagogue roles for tryptase and eq.MCP-1 are therefore investigated in this chapter by proteinase challenge of equine tracheal explants and measurement of mucus production at gross and molecular levels.

The aims of this chapter were therefore to: (a) determine if HDS can induce degranulation of equine airway luminal mast cells and if so, (b) do mast cells from control and heaves susceptible horses differ in their responsiveness to HDS and (c) investigate mast cell proteinases for potential mucus secretagogue activity using tracheal explant air-liquid interface cultures.

6.3. Materials and Methods

6.3.1. Pilot Studies

6.3.1.1. HDS Challenge of Mouse Mucosal Mast Cells

Mouse mucosal mast cells were used in a pilot study to investigate mast cell response to HDS challenge. These cells were used because they were readily available due to their use in a concurrent study and because, in contrast to mast cells in a mixed airway luminal cell population, they allowed investigation of a direct mast cell response to HDS due to the use of a pure mast cell population.

The cell culture medium TI3S was prepared by supplementation of Dulbecco's Modified Eagle's medium (DMEM, Life Technologies) with 10% heat inactivated foetal calf serum (Serotec), 100U/ml penicillin, 100µg/ml streptomycin, 2.5µg/ml fungizone, 2mM L-glutamine, 1mM sodium pyruvate, 1ng/ml recombinant human transforming growth factor-β1 (Sigma), 1ng/ml recombinant mouse IL-3 (R&D Systems), 5ng/ml recombinant mouse IL-9 (R&D Systems) and 50ng/ml recombinant rat stem cell factor (Amgen). Mouse mucosal mast cells grown from cultured bone marrow mast cell precursor cells (Brown *et al.,* 2003) were donated from another concurrent study. Cells were washed three times with TI3S to remove constitutive proteinases and a total cell count performed in duplicate using an improved Neubauer counting chamber (Fisher Scientific). Viability was assessed by 0.2% nigrosin (Sigma) exclusion. Cells were resuspended in TI3S at $5x10^6$ cells/ml and then 100µl aliquots of cells ($5x10^5$ cells) incubated in triplicate for 30min at 37°C with 100µl challenge media. Challenge media were as follows:

(a) TI3S (negative diluent control)
(b) Calcium ionophore (10^{-6}M) (Sigma) (positive control)
(c) Neat HDS
(d) 1:10 HDS
(e) 1:100 HDS
(f) 1:1000 HDS
(g) 1:10,000 HDS

Calcium ionophore was used as a positive control at a concentration of 10^{-6}M as this has previously been shown to induce high levels of degranulation of murine mast cells (Levi-Schaffer *et al.,* 1987; Thompson *et al.,* 1990; Coleman *et al.,* 1993). The HDS used contained 0.1g/ml hay dust and 1.2 x 10^8/ml particulates (<60µm diameter). This HDS had previously been produced and then characterised *in vivo* as effective in inducing a heaves response in susceptible horses (Pirie, 2002). TI3S was used as the HDS diluent. The dilution factor of the cell suspension resulted in a final HDS challenge concentration of 50mg hay dust/ml for neat HDS challenge. Serial dilutions therefore resulted in final challenge hay dust concentrations of 5mg/ml, 0.5mg/ml, 5µg/ml and 0.5µg/ml for challenges (d) to (g) respectively.

Following challenge, 20µl of cells were removed for total cell count and viability studies. The remaining cells were pelleted at 735g for 5min at 4°C and the supernatant removed and stored at -70°C for later ELISA quantitation of mouse mast cell proteinase-2 (mMCP-2). The cell pellet was washed 3 times with 1ml PBS by vortexing followed by centrifugation at 735g for 5min at 4°C. Cells were then lysed by 3 freeze-thaw cycles in 450µl 0.5M NaCl in PBS using dry ice and a 37°C water bath. Cell debris was pelleted by centrifugation at 13,000g for 5min at 4°C and the lysed cell pellet supernatant removed and stored at -70°C.

6.3.1.2. Calcium Ionophore Challenge of Equine Airway Luminal Mast Cells

The optimal calcium ionophore concentration for induction of mast cell degranulation varies among species (Wardlaw *et al.,* 1986; Sommerhoff *et al.,* 1989b; Hare *et al.,* 1998). Therefore a pilot study was performed to verify the appropriate concentration for use as a positive control challenge of equine mast cells. Furthermore, this pilot study allowed optimisation of the *in vitro* equine airway luminal mast cell challenge protocol.

Luminal airway mast cells were recovered by BAL from horses (control n=3, clinical heaves n=1) referred to R(D)SVS for clinical investigation of pulmonary disease as described in section 4.3.1.1. BALF total cell count and viability were assessed as above and differential cell counts of 500 cells performed on duplicate Leishman's

stained cytospins. Cells were pelleted by centrifugation at 200g for 10min at 4°C and the supernatant decanted. Cells were then washed twice in PBS, resuspended in Tyrode's buffer and total cell count and viability assessments repeated. Following centrifugation at 200g for 10min at 4°C, cells were resuspended in Tyrode's buffer at $6x10^5$ mast cells/ml. Aliquots of cells (100µl, $6x10^4$ mast cells) were challenged in triplicate with 300µl challenge media at 37°C for 30min as follows:

(a) Tyrode's buffer
(b) 10^{-6}M calcium ionophore
(c) 10^{-5}M calcium ionophore
(d) 10^{-4}M calcium ionophore

These challenge concentrations were based upon those reported to induce mast cell degranulation in other species. Tyrode's buffer was freshly prepared for each challenge by dissolving 4g NaCl (137mM), 0.1g KCl (2.7mM), 0.5g $NaHCO_3$ (12mM), 0.03g NaH_2PO_4 (0.37M), 0.01g $MgCl_2$ (0.1mM), 0.11g $CaCl_2$ (2mM), 0.5g glucose (0.1%) and 0.5g gelatine (0.1%) in 100ml distilled water by heating for approximately 1min in a microwave (Panasonic 800W D). When the gelatine was dissolved, 395ml distilled water was added followed by 5ml HEPES buffer (10mM, pH 7.3, Sigma). The pH was then adjusted as necessary to pH ~7.3.

Following challenge, cells were pelleted by centrifugation at 735g for 5min at 4°C and the supernatant removed and stored at -70°C until analysis. Cell pellets were washed 3 times with 1ml PBS and lysed by 3 freeze-thaw cycles with 450µl 1M NaCl in PBS. The lysed cell pellet supernatant was harvested by centrifugation at 13,000g for 10min and stored at -70°C until further analysis.

6.3.1.3. Effect of HDS on Determination of Tryptase by ELISA

As preliminary trials suggested that HDS might interfere with determination of equine tryptase by the ELISA, dilutions of 0.1g/ml HDS (neat - 1:10,000) in ELISA buffer were mixed in equal volume with 10ng/ml equine tryptase. Tryptase concentrations of samples were then determined by ELISA as previously described (4.3.2.3).

6.3.1.4. β-hexosaminidase Assay to Determine HDS Content of Supernatant and Cell Pellet

As HDS was shown to interfere with ELISA determination of equine tryptase concentrations, the relative amount of HDS retained in the supernatant and cell pellet following challenge was investigated. Dilutions of HDS (neat to 1:10,000) were made with PBS, centrifuged at 735g for 5min and the supernatant removed. Washing of the centrifuged pellet with 1ml PBS was performed 3 times and then 100µl PBS added. Neat HDS was used a positive control and PBS as a negative control. HDS had previously been shown to contain significant β-hexosaminidase activity and therefore supernatant and washed 'pellet' samples were assayed for β-hexosaminidase activity. Samples (5µl) were applied in triplicate to an ELISA plate. Substrate solution (1.3mg/ml p-nitrophenyl-N-acetyl-β-D-glucosamine in 0.1M sodium citrate pH 4.5; 50µl) was added and the plate incubated for 40min at 37°C. The reaction was then quenched by addition of 150µl 0.2M glycine solution (pH 10.7) and absorbance of samples read by a microplate reader at 405nm.

6.3.2. HDS Challenge of Equine Airway Luminal Mast Cells

6.3.2.1. Subjects and Collection of BALF

BALF was collected from control (n=6) and heaves susceptible (n=6) horses in a high state of clinical remission being used in a concurrent study at the AHT. Control horses comprised 2 mares and 4 geldings with a median (range) age of 11y (6-16y). Heaves susceptible horses comprised 2 mares and 4 geldings with a median (range) age of 15y (9-21y). Prior to sampling, all horses had been maintained at pasture and fed haylage (Marksway Horsehage) for a minimum of 3 months.

BAL was performed following sedation with 30µg/kg romifidine and 15µg/kg butorphanol using a 12mm x 1.8m flexible video colonoscope (model EC-3801L Pentax UK Ltd) connected to an EPM 3000 processor unit (Pentax UK Ltd). A lavage volume of 500ml 0.9% sterile saline at 37°C was instilled into the left dorsocaudal lung lobe in two 250ml aliquots with manual aspiration of BALF

following each instillation. BALF recovered from each instillation was pooled and kept on ice until further processing (approximately 10min). The remission status of the heaves susceptible horses and the health of the control horses was confirmed by BALF differential neutrophil counts of <4% (Dixon *et al.*, 1995b) on differential cell counts of 500 cells on Leishmans stained cytospin preparations.

Cells recovered from BAL were pelleted and washed as previously described (6.3.1.2) and then resuspended in Tyrode's buffer at $3x10^5$ mast cells/ml. A lower mast cell concentration was used compared to the calcium ionophore study in order to ensure that sufficient cells were available for all challenges. Total cell counts and viability were only assessed for these cells prior to final resuspension as the calcium ionophore pilot study had shown no significant decrease in viability with the above processing.

6.3.2.2. HDS Challenge

Aliquots of airway luminal cells (100µl, $3x10^4$ mast cells) were challenged in triplicate with 300µl challenge media at 37°C for 30min as follows:

(a) Tyrode's buffer
(b) 10^{-5}M calcium ionophore
(c) Neat HDS
(d) HDS 1:10 dilution
(e) HDS 1:100 dilution
(f) HDS 1:1000 dilution
(g) HDS 1:10,000 dilution

The HDS used in this experiment was produced at the AHT according to the method described by Pirie (2002) from hay that had previously been shown to result in marked neutrophilic airway inflammation upon natural challenge in these heaves susceptible horses, but not in the controls. This HDS contained 0.1g/ml hay dust, had a particulate count (<60µm diameter) of 286 x 10^8/ml and when nebulised, induced significantly greater neutrophil influx into the pulmonary airspace in heaves susceptible horses compared to the control group (*D. Marlin, personal*

communication). The dilution factor of the cell suspension resulted in a final challenge concentration of 75mg hay dust/ml for neat HDS challenge. Serial dilutions therefore resulted in final challenge concentrations of 7.5mg/ml, 0.75mg/ml, 75µg/ml and 7.5µg/ml for challenges (d) to (g) respectively.

Following challenge (30min, 37°C), cells were pelleted by centrifugation at 735g for 5min at 4°C. The supernatant was discarded as preliminary trials had shown that the component of HDS which interfered with ELISA determination of tryptase concentration was predominantly retained in the supernatant (sections 6.5.1.3 and 6.5.1.4). Cell pellets were washed 3 times with 1ml PBS at 735g for 5min at 4°C and lysed by 3 freeze-thaw cycles with 450µl 1M NaCl in PBS. The lysed cell pellet supernatant was then harvested by centrifugation at 13,000g for 10min at 4°C and stored at -70°C until analysis.

6.3.3. Measurement of Mast Cell Degranulation

6.3.3.1. Mouse Mast Cell Proteinase-2 ELISA

Mouse mucosal mast cell degranulation was assessed using an mMCP-2 ELISA. All steps used a volume of 50µl/well. ELISA plates (Immulon 96W, Dynatech M 129B, Thermo Life Sciences) were coated with 1µg/ml sheep anti-mMCP-2 in carbonate buffer (pH 9.6) and incubated overnight at 4°C. Wells were washed as described in section 4.3.2.1 and standards (purified mMCP-2 at 0.1-5ng/ml) and samples applied in duplicate, diluted as appropriate in ELISA buffer (section 4.3.2.1). Following incubation at 37°C for 1h, plates were washed and incubated with rat anti-mMCP-2 at 0.5µg/ml for 1h at 37°C. Plates were washed and then incubated with biotinylated rabbit anti-rat IgG (Vector Laboratories) at 1:500 dilution for 30min at 37°C. Following washing, plates were incubated with streptavidin horse radish peroxidase conjugate (Sigma, 1:1000) for 1h at 37°C. Finally, plates were washed and TMB substrate (Insight Biotechnology Ltd) added. After sufficient colour had developed, 0.18M H_2SO_4 was added to stop the reaction. Plates were read at 450nm by a microplate reader. The standard curve was generated using a linear curve fit and

sample concentrations calculated using Microplate Manager 4.0 software (Bio-Rad Laboratories).

6.3.3.2. Tryptase ELISA

Pilot study samples and lysed cell pellet supernatant samples from equine airway mast cell challenges were analysed using the tryptase ELISA. Lysed cell pellet supernatant samples were centrifuged at 20,000g for 10min at 4°C to ensure that all lysed cellular debris was pelleted. Tryptase concentrations of samples were then determined by ELISA as detailed in section 4.3.2.3.

6.3.3.3. Cytotoxicity Assay

As the cell viability of equine airway luminal cells was not assessed post-challenge, cytotoxicity assays (Cytotoxicity Detection Kit [LDH], Roche) were performed to ensure that challenge media had not caused a cytotoxic rather than a degranulation response. As cells were lysed at the end of challenge, the cell pellet supernatant should contain high concentrations of the intracellular enzyme lactate dehydrogenase (LDH). If however cytotoxicity had occurred during challenge with HDS, prior to cell lysis, LDH would have been discarded with the supernatant and consequently sample LDH would be low. The use of this assay assumes that HDS cytotoxicity would affect all cell types equally within the population, as the assay would not be sufficiently sensitive to detect isolated mast cell cytotoxicity in the mixed cell pellets.

Lysed cell pellet samples were analysed in duplicate by addition of 100µl reaction mixture (Diaphorase/NAD+ with iodotetrazolium chloride and sodium lactate) to 100µl of sample in ELISA plate wells. Following incubation at room temperature for 30min, absorbance of samples at 490nm was read by a microplate reader using a reference filter of 600nm.

6.3.4. Proteinase Challenge of Tracheal Explants

6.3.4.1. Tracheal Explant Air-Liquid Interface Culture

To investigate the potential role for mast cell proteinases as secretagogues, a pilot study was performed using air-liquid interface tracheal explant culture. Tracheal tissue was collected immediately post mortem from 3 horses euthanased at Cambridge University Veterinary School for reasons other than pulmonary disease. The left side of the neck was clipped and washed with chlorhexidine solution (Hibiscrub, Astrazeneca) and the skin reflected. The cranial 6 ribs were then removed allowing isolation and dissection of the trachea from the thorax. Tracheal tissue was immersed in DMEM supplemented with 100µg/ml gentamicin, 50µg/ml penicillin, 50µg/ml streptomycin and 50µg/ml natamycin for 4h at 37°C, with a change of medium at 2h. The tracheal tissue was then washed with antimicrobial-free DMEM for 20min, 6 times. The trachea was opened by incision of the tracheal membrane and individual tracheal rings isolated by sharp dissection. Explants of approximately 1cm length were harvested atraumatically using a single downward incision though the tissue and a fresh scalpel blade for each tracheal ring. Explants were lifted by the cartilage and placed on sterile filter paper squares on top of 1% agarose plugs in 6 well plates (Corning Life Science) with 5ml DMEM / well. Tissue viability was assessed by addition of 10µl of a white suspension of 1µm latex beads in PBS to the surface of the explant. Cilia of viable explants cleared this latex bead suspension to the anatomically proximal edge of the explant within 15min (fig. 6.1). Explants were allowed to equilibrate overnight at 37°C to restore constitutive mucus production prior to challenge.

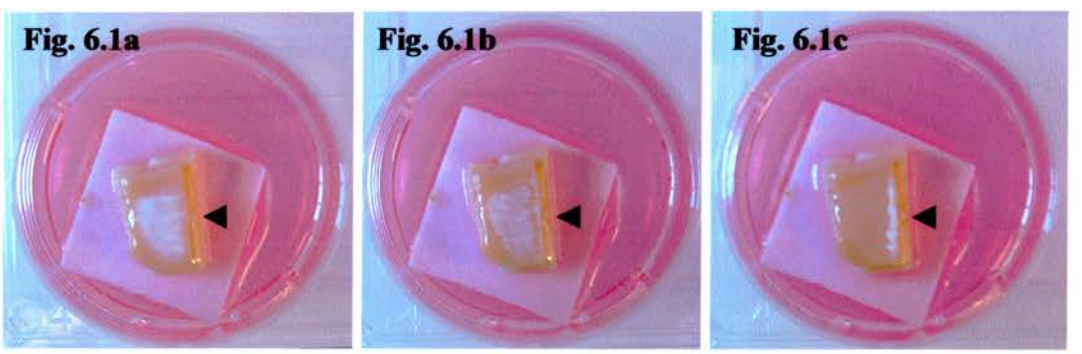

Fig. 6.1: Assessment of tracheal explant viability by clearance of 10µl of a suspension of 1µm latex beads in PBS to the anatomically proximal edge of the explant (black arrowhead) over 15min. Explants sit on sterile filter paper on top of a 1% agarose plug in 5ml DMEM. a) Latex bead suspension immediately post application, b) latex beads starting to clear proximally at approximately 5min post application, c) latex beads cleared to anatomically proximal edge of explant after 15min.

6.3.4.2. Challenge of Tracheal Explants

Following equilibration overnight, viability was again assessed by latex bead clearance. DMEM was replaced by challenge media (5ml / well) for 24h at 37°C as follows:

(a) DMEM (negative control)
(b) 10^{-10}M tryptase
(c) 10^{-9}M tryptase
(d) 10^{-8}M tryptase
(e) 10^{-10}M eq.MCP-1
(f) 10^{-9}M eq.MCP-1
(g) 10^{-8}M eq.MCP-1
(h) 10^{-8}M eq.MCP-1 + 100μg/ml soy bean trypsin inhibitor (SBTI)
(i) 100μg/ml SBTI

For challenge (h), eq.MCP-1 was incubated with the chymase inhibitor SBTI for 15min prior to addition of diluent (DMEM), to ensure inactivation of chymase prior to dilution. These challenge concentrations have previously been used to challenge serous cells in culture.

The viability of explants was re-assessed at the end of challenge. Explants were then cut in half and placed in 0.5ml PBS and 2ml RNA later. PBS samples were processed immediately whereas samples in RNA later were stored at 4°C overnight and then at -20°C until RNA extraction (approximately 2 months).

Following the first explant challenge (hereafter EXP1) it was suspected that proteinases may not be able to penetrate through the thick tracheal cartilage and therefore in the latter two experiments (hereafter EXP2 and EXP3, respectively) tracheal epithelium was stripped off the cartilage by sharp excision and used as the explant. Submersion of explants in medium for the first hour of challenge was also employed for EXP3 in an attempt to improve proteinase tissue penetration.

6.3.5. Measurement of Tracheal Explant Mucus Production

6.3.5.1. PAS Assay

A semi-quantitative measurement of explant mucus production was determined using the optical density of explant PBS washings following addition of PAS. Explants were vortexed in 0.5ml PBS for 10s to release any mucus from the epithelium. PAS (50µl) was added to 100µl of PBS washings and the OD measured at 595nm by spectrophotometer. The epithelium was then stripped off the tracheal cartilage (if present) and weighed. The absorbance of washings was then expressed as OD/mg epithelium to correct for variation in explant size. This assay is reported to have a detection limit of 1-2µg/ml mucin (Thornton *et al.,* 1989).

6.3.5.2. Quantitative Real-Time RT-PCR for Equine MUC-5AC Upregulation

Quantification of explant mRNA expression for MUC-5AC, a gel-forming mucin that is known to be upregulated in heaves horses (Gerber *et al.,* 2003) was performed on negative control explants and those deemed most likely to show a positive response, i.e. 10^{-8}M tryptase and 10^{-8}M eq.MCP-1 challenged explants. If a significant result was obtained for these maximal challenges, RNA would be extracted from the other explant challenges. Total RNA was extracted using the Qiagen RNeasy kit (section 5.3.6.2), reverse transcribed (section 5.3.7) and subject to quantitative RT-PCR for the mucin gene equine MUC-5AC and the house-keeping gene β-actin using the protocol described in section 5.3.8. Equine β-actin primers were those described in section 5.3.8 and previously published equine MUC-5AC primers for an 80bp fragment were used to quantify mucus production (Gerber *et al.,* 2003). Primers are shown in table 6.1.

Gene	**Forward primer 5' → 3'**	**Reverse primer 5' → 3'**
Equine MUC5AC	GAG GTC TTC GAG CCG TGC	GGT CAA ACA CAC AGC CTT GGT
Equine β-actin	TGG GCC AGA AGG ACT CAT AC	CTT GAT GTC ACG CAC GAT TT

Table 6.1: Sequence of primers used in quantitative real-time PCR assays of tracheal explants.

6.4. *Statistical Analyses*

6.4.1. *In Vitro* Mast Cell Challenge Pilot Studies

6.4.1.1. HDS Challenged Mouse Mucosal Mast Cells

The mMCP-2 content of the supernatant and lysed cell pellet supernatant was calculated from the proteinase concentrations determined by the ELISA. The percentage mast cell degranulation for each sample was then derived using the following equation:

$$\% \text{ degranulation} = \frac{\text{supernatant proteinase (ng)}}{\text{supernatant proteinase (ng)} + \text{cell pellet proteinase (ng)}} \times 100$$

Due to the restricted sample size of this pilot study, no statistical analyses were performed on this data.

6.4.1.2. Calcium Ionophore Challenge

The percentage mast cell degranulation for each sample was calculated as described for mouse mucosal mast cells using mean values of triplicate data. Data were not normally distributed and therefore a Friedman test for repeated measures was performed to compare degranulation among challenges followed by post hoc Dunn's test if a significant result was obtained. Supernatant tryptase content was also compared among challenges using the same analyses.

6.4.1.3. HDS Challenged Equine Airway Luminal Mast Cells

As supernatants could not be used to assess tryptase release from HDS challenged equine mast cells (sections 6.5.1.3, 6.5.1.4), assessment of degranulation could only be made using cell pellets. Little variation occurred in tryptase determination of triplicates and therefore a mean value for each set of triplicate values was calculated to give one value per challenge per horse. As basal cell pellet tryptase content was variable between horses, challenge data were expressed as the percentage of negative control cell pellet tryptase remaining post challenge and was calculated using the following equation:

$$\% \text{ negative control cell pellet tryptase remaining} = \frac{\text{challenged cell pellet tryptase (ng)}}{\text{negative control cell pellet tryptase (ng)}} \times 100$$

Data were not normally distributed and therefore Friedman tests with post hoc Dunn's test were used to examine if challenges had a significant effect on the percentage of cell pellet tryptase remaining within control and heaves susceptible horse populations. Responses of control and heaves susceptible horses to each challenge were compared using Mann Whitney tests.

6.4.2. Tracheal Explants

6.4.2.1. PAS Assay

The residuals from an ANOVA of the PAS assay data assumed a normal distribution following log transformation of the PAS assay data. Significant effects of challenge, explant experiment and their interaction on mucus production could therefore be analysed by two-way ANOVA.

6.4.2.2. MUC-5AC mRNA Expression

The crossing threshold (Ct) at which measurable fluorescence was produced was used to calculate the relative expression (RE) of MUC-5AC transcripts to β-actin transcripts using the equation:

$$RE = \frac{2^{Ct\ \beta\text{-actin}}}{2^{Ct\ MUC\text{-}5AC}}$$

RE values were log transformed which resulted in the residuals from ANOVA assuming a normal distribution. Significant effects of challenge, explant experiment and their interaction on relative expression of MUC-5AC transcripts were again analysed by two-way ANOVA.

6.5. Results

6.5.1. Pilot Studies

6.5.1.1. HDS Challenge of Mouse Mucosal Mast Cells

Cultured mouse mucosal mast cells had an initial viability of 88.3%. Mann Whitney tests revealed that total cell count and viability were not significantly different following HDS challenge (data not shown) such that HDS had no detectable cytotoxic effect on mouse mucosal mast cells. Increased degranulation, as indicated by increased supernatant mMCP-2 levels, was observed following calcium ionophore challenge, however HDS challenge did not increase degranulation above basal (negative control) levels (fig. 6.2).

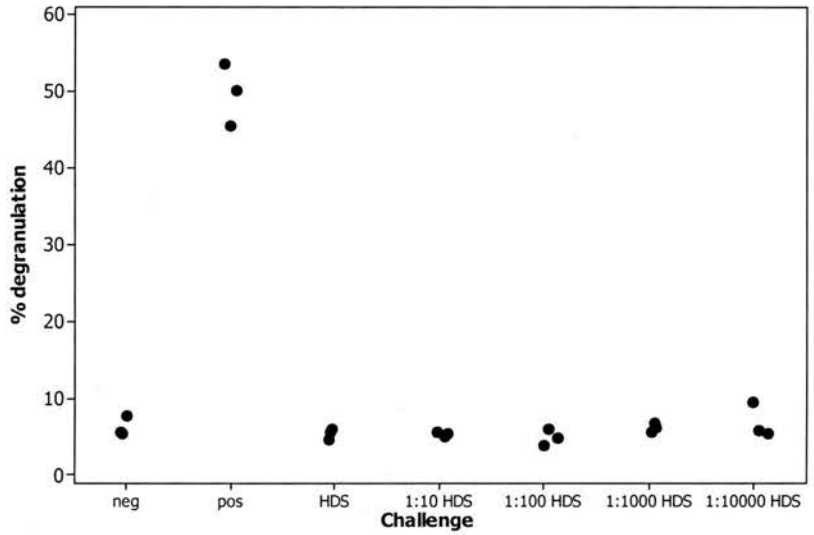

Fig. 6.2: Mouse mucosal mast cell mMCP-2 degranulation following challenge with media (neg), 10^{-6}M calcium ionophore (pos), neat HDS or HDS dilutions.

6.5.1.2. Calcium Ionophore Challenge of Equine Airway Luminal Mast Cells

Considerable inter-horse variation in basal levels of degranulation was evident, however the response of each horse to calcium ionophore showed a similar trend. There was a significant difference in degranulation among calcium ionophore challenge concentrations ($p=0.012$) with 10^{-5}M and 10^{-4}M calcium ionophore

resulting in significantly increased degranulation compared to negative control challenge ($p<0.05$) (fig. 6.3).

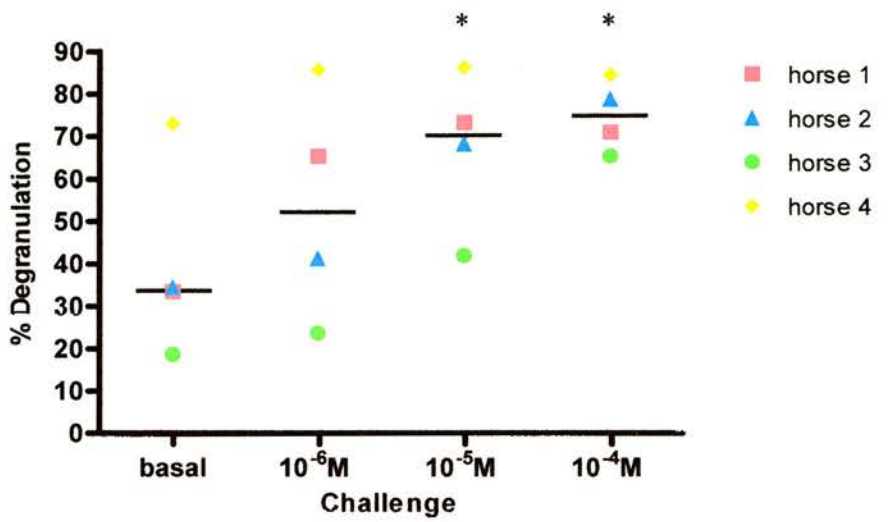

Fig. 6.3: Percentage degranulation following negative control (basal) or calcium ionophore (concentration shown) challenge of airway luminal mast cells (n=4). Challenge with 10^{-5}M and 10^{-4}M calcium ionophore led to significant increases in degranulation above basal levels ($p<0.05$). * denotes significant increase compared with basal degranulation; horizontal lines denote median values.

However, cell pellets from the 10^{-4}M calcium ionophore challenges subjectively appeared to be very fragile compared to the other cell pellets and were susceptible to fragmentation and loss during the washing process. This would result in a reduced cell pellet available for lysis and give an erroneously high degranulation value. Therefore to interpret calcium ionophore challenge data avoiding this error, supernatant tryptase content was compared among challenges. As equal numbers of mast cells were used in each challenge, theoretically basal supernatant tryptase content should be similar. However, considerable inter-horse variation was evident but again, horses showed a similar trend in response to calcium ionophore challenge. There was a significant difference in supernatant tryptase content among challenges ($p=0.006$) with supernatant tryptase content significantly increased at the 10^{-5}M calcium ionophore concentration compared with basal levels ($p<0.01$) (fig. 6.4).

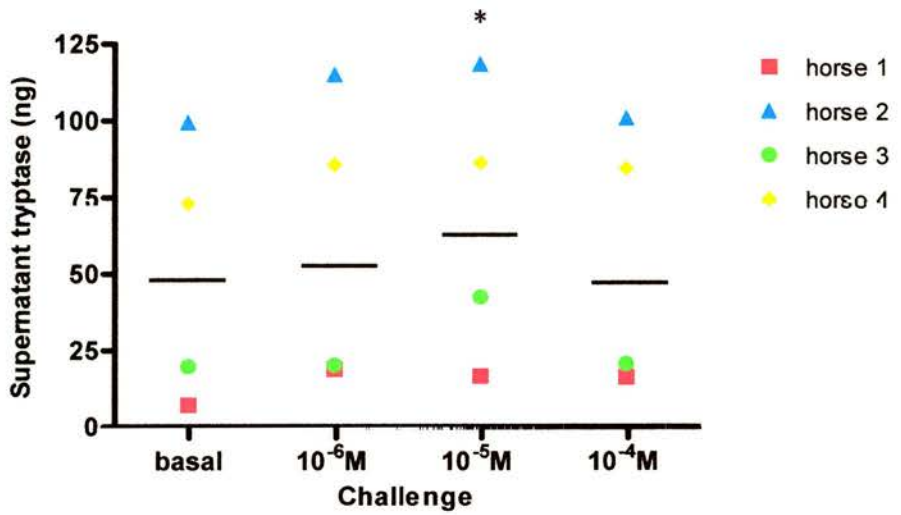

Fig. 6.4: Supernatant tryptase content (ng) following negative control (basal) or calcium ionophore challenge (concentrations shown). Challenge with 10^{-5}M calcium ionophore led to a significant increase in supernatant tryptase above basal levels ($p<0.01$). * denotes significant increase compared to basal levels; horizontal lines denote median values.

6.5.1.3. Effect of HDS on Tryptase ELISA

HDS markedly inhibited ELISA determination of equine tryptase in standards containing 10ng/ml tryptase (table 6.2). This effect decreased with dilution of HDS, until it was undetectable at a 1:10,000 dilution of HDS when tryptase quantification was equal to the sample diluted with buffer.

Sample	**Tryptase Concentration (ng/ml)**
10ng/ml tryptase	7.9
Neat HDS + 10ng/ml tryptase	< scale
1:10 HDS + 10ng/ml tryptase	< scale
1:100 HDS + 10ng/ml tryptase	3.0
1:1000 HDS + 10ng/ml tryptase	3.6
1:10,000 HDS + 10ng/ml tryptase	7.9

Table 6.2: ELISA tryptase determination of 10ng/ml tryptase diluted in equal volume with HDS dilutions. HDS significantly interfered with tryptase determination of samples. < scale = ELISA reading below the bottom of the standard curve.

6.5.1.4. HDS Content of Supernatant and Cell Pellet

Neat HDS (positive control) had high levels of β-hexosaminidase activity as determined by sample absorbance. The majority of this β-hexosaminidase activity was retained in the supernatant following centrifugation at 735g for 5min such that very little activity remained in the pellet following washing 3 times with PBS (table 6.3).

Sample	Mean Absorbance	% of Neat HDS Absorbance
Neat HDS	1.167	100
Neat HDS s/n	1.127	97
1:10 HDS s/n	0.116	10
1:100 HDS s/n	0.009	0.8
1:1000 HDS s/n	0.002	0.2
1:10,000 HDS s/n	0.004	0.4
Neat HDS pellet	0.008	0.7
1:10 HDS pellet	0.003	0.3
1:100 HDS pellet	0.002	0.1
1:1000 HDS pellet	-0.001	-0.1
1:10,000 HDS pellet	0.003	0.2
PBS	-0.001	-0.1

Table 6.3: β-hexosaminidase activity (absorbance) of neat HDS and HDS dilution supernatants (s/n) and pellets following three PBS washings. The majority of β-hexosaminidase activity was retained in the supernatant.

6.5.2. HDS Challenge of Equine Airway Luminal Mast Cells

The percentage of negative control cell pellet tryptase remaining in calcium ionophore or HDS challenged airway luminal mast cells is shown for individual horses in fig. 6.5. Whilst the response to the positive control (10^{-5}M calcium ionophore) was highly variable among horses, neat HDS challenge of airway mast cells decreased cell pellet tryptase in 4/6 control horses and 6/6 heaves susceptible horses. The median and range of percentage negative control cell pellet tryptase remaining following each challenge in control and heaves susceptible horses are shown in table 6.4.

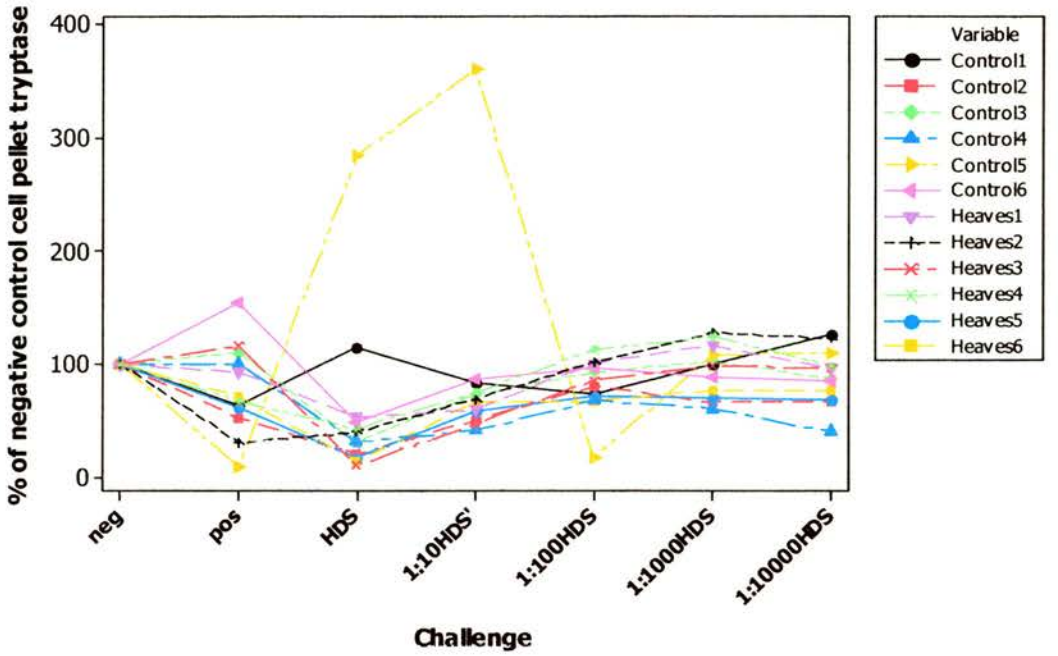

Fig. 6.5: Individual horse data (control n=6, heaves susceptible n=6) for mean percentage of negative control (neg) cell pellet tryptase remaining following challenge with 10^{-5}M calcium ionophore (pos), or HDS dilutions.

Challenge	Control Median	Control Range	Heaves Median	Heaves Range
10^{-5}M Calcium Ionophore	82.1	8.5-154.2	69.5	30.3-116.0
Neat HDS	40.3	20.2-284.1	28.5	10.1-53.0
1:10 HDS	78.3	41.1-361.1	63.0	47.1-75.0
1:100 HDS	77.1	16.4-95.7	92.8	67.5-112.4
1:1000 HDS	94.5	66.1-108.0	107.7	70.1-123.8
1:10,000 HDS	85.9	39.7-126.0	95.7	67.8-122.3

Table 6.4: Median and range of cell pellet tryptase remaining in challenged cell pellets from control (n=6) and heaves susceptible (n=6) horses expressed as a percentage of negative control.

From fig. 6.5 it can be seen that control horse 5 is an obvious outlier in response to challenge. Statistical analyses were therefore performed following exclusion of data from this horse. There was a significant difference in the percentage cell pellet

tryptase remaining following challenge in heaves susceptible horses ($p<0.001$) but not control horses ($p=0.09$). However, as exclusion of control horse 5 resulted in analysis of only 5 control horses, this non-significant result may be due to a restricted sample size. Heaves susceptible horses had significantly decreased cell pellet tryptase compared to the negative control following challenge with neat HDS (75mg/ml) ($p<0.01$) and 1:10 HDS (7.5mg/ml) ($p<0.05$) (fig. 6.6). There was no significant difference in response to any challenge between control and heaves susceptible horses ($p>0.20$).

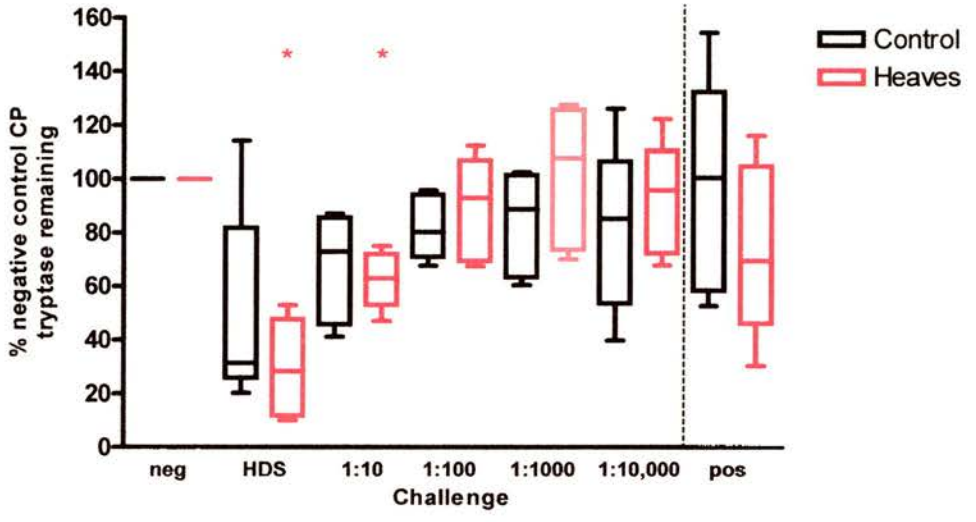

Fig. 6.6: Percentage of negative control (neg) tryptase remaining in 10^{-5}M calcium ionophore (pos) or HDS (dilutions shown) challenged cell pellets of control (excluding control horse 5, n=5) and heaves susceptible (n=6) horses. * denotes significantly different to the negative control ($p<0.05$).

6.5.2.1. Cytotoxicity Assay

The majority of samples had very low absorbance values indicative of low LDH values. There was no evidence of a dose response to HDS dilutions (fig. 6.7). These low LDH values may be the result of cell losses during washing or cell death prior to challenge. Alternatively, equine airway luminal cells may have inherently low LDH levels.

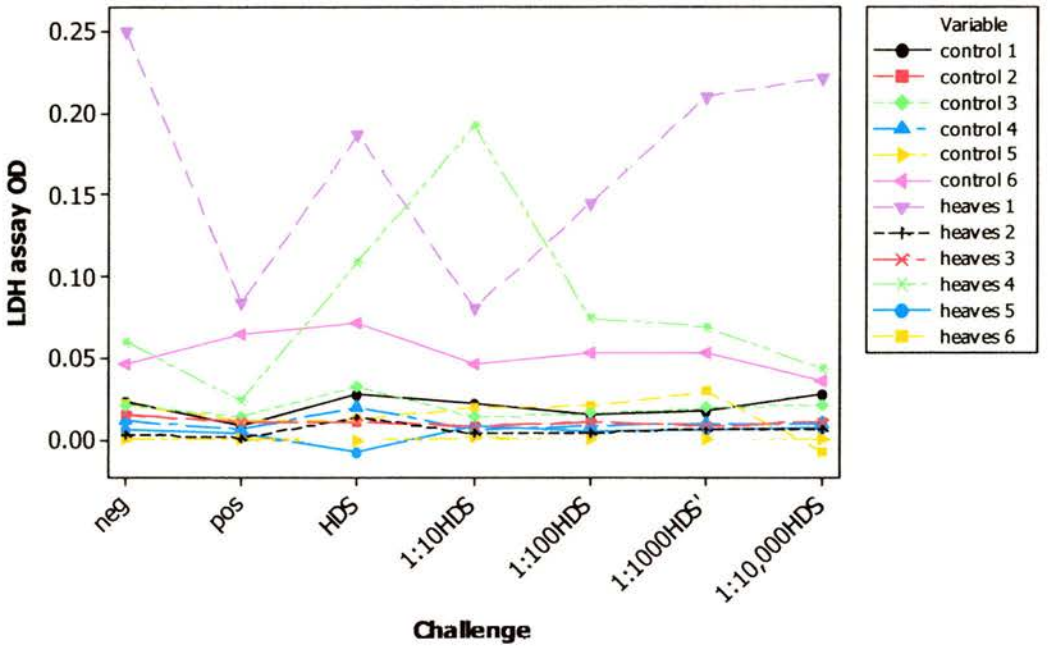

Fig. 6.7: Individual horse data for cytotoxicity assays of negative control (neg), calcium ionophore (pos) or HDS (dilutions shown) challenged cell pellets for control (n=6) and heaves susceptible (n=6) horses.

The relationship between cell pellet tryptase and LDH with different challenges was investigated. If HDS had a cytotoxic effect, low tryptase and low LDH (OD) values would be expected in neat HDS challenged cell pellets. In contrast, negative control and low HDS dilution challenged cell pellets would have high tryptase and high LDH giving a positive correlation between cell pellet tryptase and LDH. No clear relationship between cell pellet tryptase and LDH was evident. There appeared to be two populations of samples, however these were not defined by challenge (fig. 6.8) including when control and heaves susceptible horse populations were examined independently.

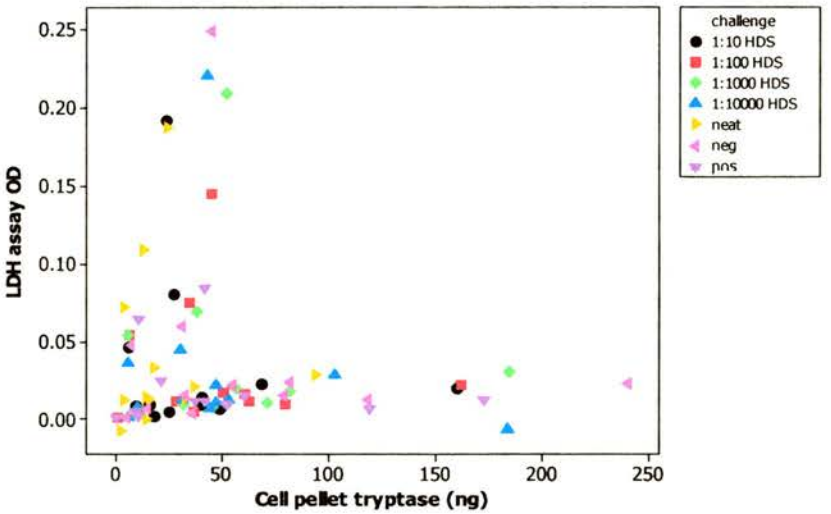

Fig. 6.8: Scatterplot of cell pellet tryptase content (ng) and OD (representing LDH content) of samples.

Sample ODs were at the lower limit of sensitivity for the cytotoxicity assay and the microplate reader. Therefore, as no consistent evidence of a cytotoxic effect was apparent, further analysis of this data was not performed.

6.5.3. Tracheal Explant Challenge

6.5.3.1. Viability of Explants

One explant from the 10^{-9}M tryptase challenge in EXP2 became infected with micro-organisms at 48h and had to be discarded. All other explants maintained good viability throughout the course of the experiment.

6.5.3.2. PAS Assay

There was no significant difference in mucus production, as measured semi-quantitatively by PAS assay of explant washings, among explant systems ($F_{2,53}$=1.7, p=0.19) or among challenges ($F_{8,53}$=1.1, p=0.38) (fig 6.9). Furthermore, there was no significant interaction between explant system and challenge ($F_{16,53}$=1.6, p=0.12).

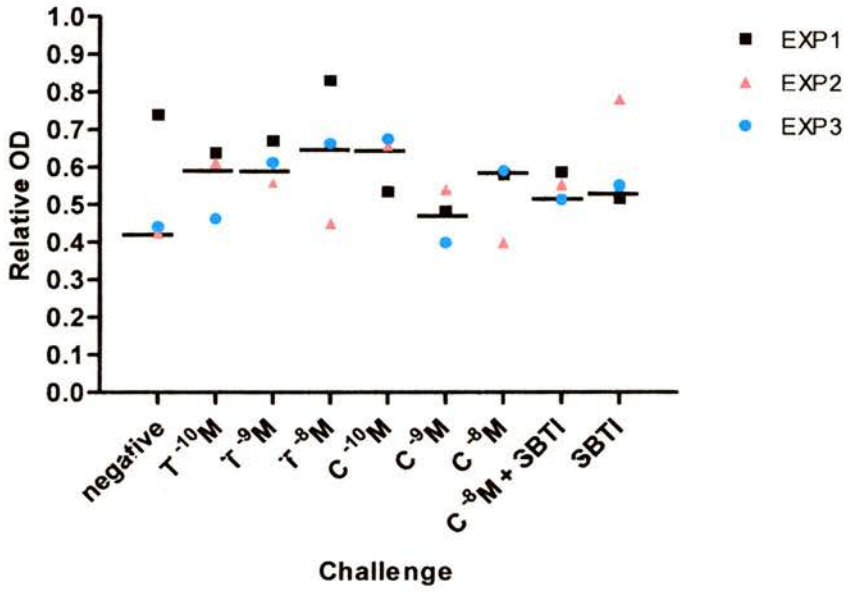

Fig. 6.9: PAS assay relative OD (absorbance / mass of epithelium) values for proteinase challenged explants. Challenge concentrations are shown on x-axis. T = tryptase, C = eq.MCP-1, SBTI = soy bean trypsin inhibitor (100µg/ml), negative = DMEM challenged explants. Horizontal lines denote median values.

6.5.3.3. MUC-5AC mRNA Expression

No RNA could be isolated from tryptase challenged explants. Inexplicably the RNA appeared to have degraded prior to extraction.

For the limited number of samples available, neither explant experimental design nor challenge with 10^{-8}M eq.MCP-1 had a significant effect on relative MUC-5AC transcript expression ($F_{2,17}$=2.0, p=0.18 and $F_{1,17}$=0, p=0.96 respectively) (fig. 6.10). Again, there was no significant interaction between explant system and challenge ($F_{2,17}$=2.1, p=0.17).

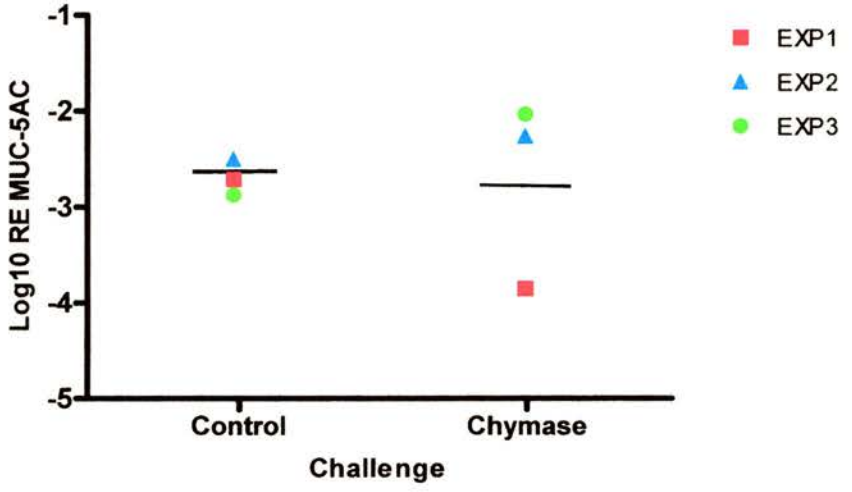

Fig. 6.10: Log10 relative expression (RE) of MUC-5AC in tracheal explants (EXP 1-3) challenged with DMEM (control) or 10^{-8}M eq.MCP-1 (chymase). Horizontal lines denote median values.

6.6. Discussion

6.6.1. Calcium Ionophore Challenge of Mast Cells

Percentage degranulation data for equine airway luminal mast cells appeared to show a dose dependent response to calcium ionophore with little degranulation in response to 10^{-6}M, but significant degranulation with both 10^{-5}M and 10^{-4}M challenges. In contrast, cultured mouse mucosal mast cells showed pronounced degranulation following 10^{-6}M calcium ionophore challenge. These findings are consistent with other reports of variation in optimal calcium ionophore concentrations for induction of mast cell degranulation among species (Wardlaw *et al.,* 1986; Sommerhoff *et al.,* 1989b; Hare *et al.,* 1998). The percentage degranulation data for equine airway luminal mast cells may be misleading however, due to an erroneously high degranulation index for 10^{-4}M calcium ionophore challenge, which appeared to be caused by cell pellet loss during washing, as described in section 6.5.1.2. Supernatant tryptase content was therefore considered to more accurately represent degranulation in response to calcium ionophore challenge. Using this data, significant degranulation of equine airway luminal mast cells only occurred following challenge with 10^{-5}M calcium ionophore, in agreement with a previous study by Hare *et al.*

(1999). This waning response to higher concentrations of calcium ionophore has been previously reported and is suggested to be due to over-saturation of the adenyl-cyclase pathway by calcium or the release of mast cell inhibitory agents from other inflammatory cells (Wardlaw *et al.,* 1986; Sommerhoff *et al.,* 1989b; Hare *et al.,* 1998). For further experiments, challenge with 10^{-5}M calcium ionophore was therefore chosen as the positive control.

Levels of basal airway luminal mast cell degranulation were highly variable among horses, however it is difficult to comment upon this due to the small sample size of this pilot study. As horses used in this pilot study were clinical cases from the R(D)SVS hospital, their management was unknown and may have been very varied, possibly accounting for some of the inter-horse variability in basal degranulation. However, horses used in the HDS mast cell challenge study, which all had identical management, also displayed variable basal cell pellet tryptase content, which may therefore reflect inherent individual variation in basal degranulation. This variability appeared to be independent of heaves status.

The level of basal degranulation was considerably higher in equine airway luminal mast cells compared to that of the cultured mouse mucosal mast cells. Airway luminal mast cells are a dynamic, heterogeneous cell population which may be responding to their surrounding microenvironment, and have undergone physiological or immunological degranulation during their migration into the air spaces, in contrast to cultured cells which represent a comparatively homogenous and quiescent cell population. Furthermore, the presence of other cells in the mixed airway luminal cell population may provide constant low level stimulation of mast cells increasing their basal degranulation.

Response to 10^{-5}M calcium ionophore was very variable among horses in the HDS study with 5/12 horses (3 control, 2 heaves susceptible horses) showing no response to this positive control challenge. In fact, all equine mast cells challenged appeared relatively insensitive to calcium ionophore despite a significant dose dependent effect being evident in the pilot study. Similarly, wide inter- and intra-horse

variability in mast cell and basophil induced degranulation has been reported previously (Kings and de Weck, 1980; Hare *et al.,* 1998) and is also apparent in other species (Lichtenstein, 1975; Flint *et al.,* 1985b; Wells *et al.,* 1986). There was no significant difference between control and heaves horses in their response to this 10^{-5}M calcium ionophore positive control challenge as previously reported by Hare *et al.* (1999). Similarly, there is no significant difference in basal or calcium ionophore induced BALF mast cell degranulation in mongrel (control) and innately hyper-responsive Basenji greyhounds (Sommerhoff *et al.,* 1989b), control and asthmatic humans (Casolaro *et al.,* 1989) or control and *Ascaris suum* sensitised monkeys (Wells *et al.,* 1986). In contrast however, other authors have reported increased spontaneous (basal) and induced histamine release from human asthmatics compared to control individuals (Flint *et al.,* 1985a) and Basenji greyhounds compared to mongrel dogs (Hirshman *et al.,* 1986).

6.6.2. Effect of HDS on Tryptase ELISA

HDS had a dramatic effect on determination of tryptase concentrations by the ELISA. Neat HDS and a 1:10 dilution of HDS rendered tryptase undetectable. This effect decreased with serial dilution of HDS until tryptase quantification of a sample mixed with a 1:10,000 dilution of HDS was equivalent to that of a sample diluted with buffer.

The components interfering with the ELISA were retained in the supernatant following centrifugation of HDS suggesting that soluble factors, rather than particulates, are responsible for this effect. HDS has previously been found to contain a 28kDa serine proteinase and 85 and 160kDA metalloproteinases (Pirie, 2002). Although it is unlikely that these proteinases competed with tryptase for binding sites, they may have inactivated the capture antibody, degraded tryptase or otherwise interfered with the ELISA. Alternatively, other HDS components may have interfered with tryptase binding in some way.

This interference made it impossible to quantify tryptase concentrations of mast cell challenge supernatants. Instead, a measure of cell pellet degranulation was determined by expressing the amount of tryptase in the challenged cell pellet as a percentage of the control cell pellet tryptase.

The source of β-hexosaminidase activity in HDS is unknown. HDS contains fungal spores, bacteria and storage mites (Pirie *et al.,* 2002b) all of which contain numerous enzymes (Childs and Bowman, 1981; Zakharova, 1998; Stewart *et al.,* 1998). It is therefore speculated that these mite, bacterial and/or fungal enzymes may have been the main source of β-hexosaminidase activity in the HDS.

6.6.3. HDS Challenge of Mast Cells

This study is the first to report *in vitro* challenge of equine airway mast cells with HDS. Prior studies have investigated *in vitro* fungal antigen challenge of equine airway luminal mast cells, however *in vivo* studies have shown that these fungal challenges do not fully represent the natural hay / straw challenge required to initiate a heaves response (McGorum *et al.,* 1993d). HDS challenge was therefore used to more closely mimic *in vivo* natural challenge. Mast cells from both control and heaves susceptible horses showed evidence of a non-cytotoxic degranulation in response to HDS challenge. Significant degranulation was observed in airway mast cells from heaves susceptible horses challenged with neat HDS (75mg/ml) and a 1:10 dilution of HDS (7.5mg/ml). As similar dose response patterns were observed in both control and heaves susceptible horses, the lack of significant degranulation in control horses was considered likely to be the result of low sample size due to the exclusion of control horse 5 as an outlier. Indeed, there was no significant difference between control and heaves horses in their response to any challenge. This indicates that the increased BALF concentrations of tryptase noted in heaves horses does not appear to be due to mast cells from heaves horses having increased responsiveness to HDS.

Although challenge of equine airway luminal mast cells with HDS has not previously been reported, the response of equine basophils to mould and dust extracts has been

studied. Peripheral basophils from heaves horses showed degranulation and histamine release at lower concentrations of mould extracts than those from control horses (Gerber *et al.,* 1982). However, extracts of hay and straw dust did not result in significant basophil degranulation in either control or heaves affected horses (Dirscherl *et al.,* 1993). This lack of peripheral blood basophil response to hay and straw dust extract does not preclude a compartmentalised airway mast cell response. This is demonstrated by the fact that airway luminal mast cells from asthmatics show increased histamine release with anti-IgE compared to controls in the absence of any peripheral blood basophil response (Flint *et al.,* 1985a). Alternatively, the dust extract used by Dirscherl *et al.* (1993), for which details are not given, may not have been appropriate and would ideally have been tested for its ability to evoke a positive response in heaves susceptible individuals following inhalation prior to use in *in vitro* challenge, as was performed in the present study.

Similar levels of mast cell response to HDS were observed in both control and heaves susceptible horses in this study. However, in Chapter 4 it was determined that heaves horses had significantly increased BALF tryptase concentrations compared to controls. This apparent contradiction could be explained by the increased number of intra-epithelial mast cells present in the airways of heaves horses (Chapter 5) such that there are considerably more mast cells available to respond to challenge in heaves horses. Alternatively, the *in vitro* HDS challenge may have been an excessively severe, acute challenge compared to sub-acute or chronic lower level *in vivo* challenges.

This similar responsiveness of mast cells from control and heaves susceptible horses may also suggest that the observed HDS induced mast cell degranulation is unlikely to be via allergen specific crosslinkage of IgE to high affinity receptors (FcεRI), further questioning the role of type I hypersensitivity in equine heaves. However, it is also possible that both control and heaves horses were sensitised to allergens in the hay and straw and underwent IgE-mediated mast cell degranulation, but that subsequent events differ between control and heaves horses such that controls do not respond clinically. This dissociation between mast cell degranulation and clinical

response has previously been described for adenosine challenge of control and asthmatic subjects which caused similar mast cell mediator release in both groups, but bronchoconstriction only in asthmatic individuals (Crummy *et al.,* 2004).

In contrast to the current study, Hare *et al.* (1999) reported increased histamine degranulation in heaves horses compared to controls following *in vitro* challenge of airway luminal mast cells with extracts of fungi commonly found in hay and straw. It is therefore possible that mast cells may be activated by multiple pathways by different dust components. Although increased BALF levels of IgE to *Faeni rectivirgula* and *Aspergillus fumigatus* have been reported in heaves horses compared to controls (Halliwell *et al.,* 1993; Schmallenbach *et al.,* 1998), fundamental verification of the involvement of type I hypersensitivity in the pathogenesis of equine heaves is still lacking. There is some evidence from immunohistochemical IgE staining of lung tissue from control and heaves horses to suggest that different phenotypes of equine heaves may occur, such that IgE mediated responses play a role in some individuals and not others (van der Haegen *et al.,* 2001). In further support of this hypothesis, increased serum IgE to grain dust and grass pollen allergens has been reported in a proportion of heaves susceptible horses (*L. Monreal, personal communication*). Considerable inter-horse variation in response to *in vitro* equine airway luminal mast cell challenge with fungal antigens (Hare *et al.,* 1999) could also be explained by phenotypic diversity of heaves horses with Type I hypersensitivity to fungal allergens existing in some heaves phenotypes.

It is now recognised that non-immunological mast cell degranulation may also result from activation via neuropeptides, complement, endotoxin, bacterial lectins, cytokines, adenosine, changes in osmolality and tryptase itself (Church *et al.,* 1989; Bingham and Austen, 2000; Holgate, 2000; Hart, 2001; Crummy *et al.,* 2004). HDS may therefore induce mast cell degranulation via one of these alternative pathways. Although grain dust can activate complement (Olenchock *et al.,* 1980), histamine release from grain dust challenged human lung fragments was shown to be independent of complement activation, cell cytotoxicity and endotoxin (Chan-Yeung *et al.,* 1987). Antigens of *Faeni rectivirgula* are also able to activate complement via

the alternative pathway and to induce pulmonary inflammation without antigen-antibody interaction (Slauson and Hahn, 1980). Furthermore, some *Faeni rectivirgula* antigens are proteolytic enzymes with a trypsin or chymotrypsin-like effect which may have both a direct inflammatory and antigenic effect in the lung (Slauson and Hahn, 1980).

The presence of other cells in the luminal airway mixed cell suspension certainly allows the possibility of indirect mast cell degranulation requiring co-factors such as cytokines from macrophages and lymphocytes, the predominant cells present due to horses being either controls or heaves horses in remission. Indeed, grain dust has been shown to indirectly enhance degranulation of guinea pig lung mast cells stimulating the release of a mast cell degranulating factor from splenic cells immunised against grain dust allergens (Alam *et al.,* 1988). The fact that the pure population of mouse mucosal mast cells failed to degranulate following incubation with HDS may also support indirect mast cell degranulation. However, the mouse mucosal mast cells may have been incapable of an IgE-mediated response simply due to lack of prior exposure and hence sensitisation to allergens in the hay dust. Mucosal mast cells should be representative of airway luminal mast cells which are believed to be of the mucosal type (Wenzel *et al.,* 1988), however they may not be representative of fully functioning pulmonary mast cells further matured by their microenvironment. Alternatively, species differentiation in mast cell susceptibility to HDS may occur. Isolation of a pure population of mast cells from BALF would be required to investigate the role of direct or indirect airway mast cell degranulation further. While simian mast cells have been purified from BALF using density gradient centrifugation (Wells *et al.,* 1986), this technique was unable to resolve mast cells from eosinophils and neutrophils. Similarly, attempts to isolate mast cells from human lung digests (Alam *et al.,* 1988) resulted in only 30% purity of mast cells, which is insufficiently pure to allow study of mast cells without contamination of other cells. Isolation of purified mast cells from equine BALF or lung tissue has not been described.

The mechanism by which dust can induce mast cell degranulation therefore remains elusive. The current study and the reports cited suggest that at least part of the response is likely to be via non-immunological means. Basic peptides (Shanahan *et al.,* 1984; Chan-Yeung *et al.,* 1987) and the lectin-like activity of grain dust (Alam *et al.,* 1988) have been proposed as possible pathways but require validation. Further attempts to elucidate the mechanism of HDS-induced mast cell degranulation could include investigation of response following fractionation of HDS or endotoxin removal and the use of antibodies against IgE or cytokines such as TNF-α.

Grain dust stimulated mast cell degranulation has been investigated in several occupational asthma models. Dose dependent degranulation occurred in guinea pig lung mast cells following challenge with 0.03 to 0.25mg/ml grain dust (Alam *et al.,* 1988) and in lung fragments from control human subjects following 0.1-0.5mg/ml exposure (Chan-Yeung *et al.,* 1987). Grain dust challenge of occupational asthmatics also results in enhanced basophil histamine release compared to controls with a dose-dependent response observed from 0.01 to 0.1mg/ml grain dust (Park *et al.,* 1999). These studies have reported significant mast cell degranulation at doses considerably lower than the current study. Grain dust may be more potent than hay dust in stimulating mast cell degranulation, or alternatively, preferential histamine degranulation may mean that this index of degranulation, as measured in the occupational asthma studies, is not reflective of tryptase degranulation. The biological significance of *in vitro* dust induced mast cell degranulation is difficult to speculate upon as dust concentrations encountered by airway luminal and intra-epithelial mast cells naturally *in vivo* are unknown. It is likely that natural challenge *in vivo* presents a lower level challenge over a longer period of time, however prolonged exposure to this environment may achieve similar dust concentrations in the pulmonary airspace as those from acute, high concentration nebulisation or *in vitro* HDS challenges. The aforementioned grain dust stimulated release of a mast cell degranulating agent from splenic cells occurs at much lower concentrations than direct dust induced mast cell degranulation. It has therefore been suggested that this indirect influence on degranulation may have greater biological relevance.

Increased serum neutrophil chemotactic activity is present in occupational asthmatics following grain dust challenge (Park *et al.,* 1999). This chemotactic activity has been shown to consist of heat-stable and heat-labile components, with the mast cell proposed to be the source of the heat-stable activity (Nagy *et al.,* 1982). This is of interest given that the primary cellular response in exacerbation of heaves is neutrophil influx into the pulmonary airspace and, given the current findings of HDS induced equine airway luminal mast cell degranulation, warrants further investigation in the horse.

In Chapter 5 it was argued that BALF mast cells may be a senescent cell population due to lack of upregulation of tryptase transcripts following a 48h hay / straw challenge in heaves horses. However, equine airway luminal mast cells are clearly capable of degranulation in response to non-specific degranulating agents such as calcium ionophore and to HDS. Therefore, whilst it appears from the time-points examined that these luminal mast cells may not be able to upregulate mRNA transcripts in response to challenge, contribution to increased BALF tryptase concentrations by degranulation could occur. As previously discussed, it is believed that intra-epithelial mast cells may play a substantial role in response to inhaled allergens and particulates and this contribution cannot be inferred by studying only luminal mast cell responses. Despite these limitations in fully replicating an *in vivo* challenge, this *in vitro* system demonstrated significant mast cell degranulation following HDS challenge of mast cells. This system will therefore be useful for further investigating the mast cell response to HDS challenge.

6.6.4. Tracheal Explant Challenge

No increase in mucus production or upregulation in mucin gene expression was evident following proteinase challenge of tracheal explants as measured by PAS assay or MUC-5AC transcript expression. All explants were derived from control horses with no history of respiratory disease. It is possible, but unlikely, that mucus cells in heaves horses may have exaggerated responses to secretagogues such that a response was not observed with control subjects.

Challenge with $4x10^{-6}$M trypsin stimulates a three-fold increase in airway secretion from tracheal ring organ culture in hamsters (Niles *et al.,* 1986). In contrast, 10^{-8}M tryptase had no effect on mucus secretion from cultured bovine submucosal gland serous cells (Sommerhoff *et al.,* 1989a). Although this divergence in response may be related to a difference in challenge proteinase concentrations, it may also be explained by the relative contributions of submucosal glands and goblet cells to airway secretions in different species. Rodents have few submucosal glands and primarily secrete mucus from goblet cells in the epithelial surface whereas, in humans and most other mammals, the majority of airway secretion is submucosal gland derived (Sommerhoff *et al.,* 1989a). The horse is somewhat unusual in that there are substantial contributions from both systems but goblet cell contribution is reported to be greater than that of the submucosal glands in both control and heaves horses (Nicholls, 1978; Hall *et al.,* 1998). As goblet cells are located in the epithelial compartment, it would be presumed that local proteinase concentrations should have been high following 1h submersion in challenge medium in EXP3. Furthermore, 1h challenge is reported to be sufficient to measure a mucus degranulation response from goblet cells in a tracheal ring organ culture (Niles *et al.,* 1986). The air-liquid interface culture may not have provided a suitable model of the equine airway for mucus production with other essential co-factors possibly being absent. Alternatively, tryptase may have dissociated into inactive monomers following reduction in salt concentration on dilution with DMEM or by the heparin scavenging action of lactoferrin in the tracheal mucus.

Chymase markedly stimulates mucus secretion from cultured bovine airway submucosal gland serous cells with a 10^{-8}M chymase solution increasing mucus production by >1500% (Sommerhoff *et al.,* 1989a). This secretory response had a threshold of 10^{-10}M, and was blocked by the chymase inhibitors soy bean trypsin inhibitor and chymostatin. As such, chymase is the most potent secretagogue for cultured airway gland serous cells identified so far with respect to both threshold concentration and magnitude of response (Sommerhoff *et al.,* 1989a). Although chymase concentrations of 10^{-10}M to 10^{-8}M stimulated dose-dependent mucus release

from cultured airway gland serous cells, we observed no response with these concentrations with equine tracheal explants. The chymase used by Sommerhoff *et al.* (1989a) was an α-chymase and therefore may not be analogous to eq.MCP-1 which is a β-chymase. An equine α-chymase homologue, which may have similar secretagogue properties, has not yet been identified. Furthermore, application of chymase directly to cultured cell monolayers as used in the study by Sommerhoff *et al.* (1989a) will obviously result in high chymase concentrations local to the mucus secreting cells, whereas use of the same concentrations in explants requires the proteinase to pass through tissue to reach submucosal glands. Not only may this passage through the tissues be a physical impediment, it may also result in much lower local concentrations at the submucosal glands than those applied. Therefore although air-liquid interface explant culture allows *in vitro* manipulation of airway tissue in the most physiologically natural environment, this system does not allow direct application of proteinases to the submucosal gland area. Consequently, improved proteinase penetration of the tissues was attempted by removal of cartilage in EXP2 and 3 and an initial 1h submersion in challenge medium for EXP3. However, even luminal challenge of explants requires the proteinases to pass through the covering mucus layer which contains antiproteinases (Dixon, 1992) and therefore may have inhibited proteinase activity. Suitable equine airway gland serous cell culture lines, which would have allowed high local proteinase concentrations possibly more representative of *in vivo* periglandular mast cell degranulation, are not presently available.

6.7. Conclusion

This study has shown that HDS causes significant *in vitro* airway luminal mast cell degranulation from heaves horses at challenge concentrations of 75 and 7.5mg/ml. Control horses appeared to respond in a similar dose dependent manner, although statistical significance was not achieved. Control and heaves horses did not differ in their response to HDS and therefore this HDS induced luminal mast cell degranulation is believed to be non-IgE mediated unless both populations of horses were sensitised. Essential co-factors for degranulation may have been provided by

macrophages or lymphocytes in the mixed luminal cell population. The availability of a greater number of mast cells in the epithelial compartment may be the determinant of increased BALF tryptase concentrations in heaves horses but not controls. Alternatively, there may be divergence of mast cell mediator release and subsequent inflammatory events.

We were unable to demonstrate a significant increase in mucin gene expression or mucus production in tracheal explants following proteinase challenge. It is possible that equine mucus secreting cells may not be responsive to mast cell proteinases. However, it was considered more likely that insufficient concentrations of proteinases were achieved locally at the mucus glands due to either poor tissue penetration or inactivation of proteinases.

Chapter 7: Concluding Addendum

This series of studies has investigated the role of mast cells and mast cell serine proteinases in equine heaves and has provided evidence for their participation in the pathogenesis of the pulmonary inflammatory response. The key findings are summarised below.

Cloning and sequencing of equine tryptase allowed rationalisation of its trypsin-like enzymic activity. Uniquely among tryptases, equine tryptase contains an alanine substitution at residue 216, which confers increased arginine specificity. This finding may have biological significance in inflammatory pathways, affecting its spectrum of activity compared to tryptases in other species, for example by modifying cleavage of fibrinogen-β chains. Determination of this tryptase cDNA sequence also enabled design of molecular tools to probe transcription of equine tryptase in airway luminal and tissue mast cells in control and heaves affected horses. Attempted cloning and sequencing of eq.MCP-1 resulted in the sequencing of a novel chymase similar to equine mastocytoma derived eq.MCP-1. As the cloned sequence shared strongest identity with lymphocyte derived granzyme proteinases, the cellular origin of the cloned proteinase could not conclusively be determined to be the mast cell.

Clinical heaves horses had significantly elevated BALF tryptase concentrations compared to controls or heaves horses in remission, providing *in vivo* evidence for mast cell degranulation in heaves horses following challenge. Horses with other pulmonary diseases also showed evidence of mast cell degranulation suggesting that this may be a general component of the equine pulmonary inflammatory response rather than a response restricted to heaves. Very little eq.MCP-1 was detected in BALF from any of the horses sampled. Likewise, whilst tryptase immunoreactive mast cells were common in equine lung tissue and BALF cytospins, eq.MCP-1 immunoreactive cells were scarcely observed. Therefore the chymase eq.MCP-1 appears unimportant in the lungs of healthy or heaves affected horses. Histamine was degraded in many BALF samples that had been archived and therefore could not be

measured reliably. It appears that BALF histamine concentrations should be analysed or stabilised immediately post collection to avoid this degradation, or alternatively, other more stable mediators such as tryptase be used as a measure of mast cell degranulation.

Increased numbers of tryptase positive mast cells were observed in the airway epithelium of heaves horses compared to controls following exposure to hay and straw. Total numbers of mast cells in the airway tissue remained the same and therefore this appeared to be redistribution rather than recruitment of mast cells. There was no difference between control and heaves horses in the number of tryptase positive mast cells in the airway lumen such that elevated BALF tryptase concentrations occur in heaves horses without a concomitant increase in the number of luminal mast cells. Together, these findings suggest that intra-epithelial mast cells may be an important source of BALF tryptase. As samples were not available from heaves horses in full remission, it is unclear whether increased intra-epithelial mast cells are a persistent feature of the heaves airway, or whether they are recruited to the epithelium following inciting challenge. Similarly, whilst tryptase mRNA transcripts in airway luminal cell pellets were not increased following challenge of heaves susceptible horses, bronchiolar tissue transcripts were significantly down-regulated in heaves horses in the early resolution phase of disease. This down-regulation of tryptase transcripts following removal of challenge is likely to be at least partly responsible for the decrease in BALF tryptase concentrations as heaves horses enter disease remission, to values not significantly different from control horses. These results again suggest that tissue mast cells are more important in response to challenge than luminal mast cells. Enumeration of mast cells in epithelial brushings from horses during heaves remission and at serial time points post challenge would allow definition of epithelial mast cell population dynamics in response to challenge. Furthermore, sequential measurement of tryptase transcript expression in airway brushings or biopsies from control and heaves susceptible horses before, during and post challenge would allow further clarification of the molecular response of tissue mast cells to challenge.

The number of mast cells in control and heaves affected horses were not significantly different in other airway tissue compartments, including airway smooth muscle. This indicates that, in contrast to human asthma, appreciable mast cell recruitment to the smooth muscle does not occur following challenge of heaves horses, at least within the time period studied. Consequently, mast cell mediators may not play a significant role in bronchospasm in equine heaves.

In vitro HDS challenge of mixed cell populations harvested from the airway lumina induced significant mast cell degranulation in heaves susceptible horses following challenge with concentrations of 75mg/ml and 7.5mg/ml HDS. However, there was evidence of dose dependent degranulation in response to HDS in both control and heaves susceptible horses. The increased number of intra-epithelial mast cells in heaves horses available to respond to inhaled challenges may explain the divergence of mast cell responses in control and heaves horses to *in vitro* and *in vivo* challenges. Alternatively, acute, severe *in vitro* challenges may represent an excessive challenge compared to sub-acute or chronic lower level *in vivo* challenges. The presence of other cells (predominantly macrophages and lymphocytes) in the challenged airway luminal cell population did not allow differentiation between HDS-induced direct and indirect mast cell degranulation. The use of isolated mast cell populations purified from lung digests or airway luminal cell pellets would aid further understanding of this degranulation response. Alternatively, depleting airway luminal cells of other cell types prior to HDS challenge would potentially allow identification of cells providing essential co-factors. The evidence of HDS-induced degranulation in both control and heaves susceptible horses may suggest a role for non-IgE mediated mast cell degranulation. However, it is possible that both IgE and non-IgE mediated degranulation occur, with phenotypic diversity of heaves horses modulating the role that type I hypersensitivity plays in their response to challenge. Treatment of control and heaves susceptible horses with monoclonal anti-IgE antibody during *in vivo* dust challenge may further elucidate the role of type I hypersensitivity in equine heaves.

The demonstration of mast cell degranulation in heaves susceptible horses following *in vivo* challenge warrants further study into the potential role of mast cell proteinases in the pathophysiology of equine heaves. Whilst we were unable to demonstrate mucus hypersecretion or MUC-5AC gene upregulation following *in vitro* proteinase challenge, this may reflect limitations in the *in vitro* model rather than precluding proteinase induced mucus secretion in heaves. Other potential effects of tryptase include smooth muscle spasm, airway remodelling, potentiation of airway inflammation and direct and indirect neutrophil chemotaxis. Paradoxically, neutrophil recruitment by mast cells may ultimately aid inactivation or clearing of tryptase from the airway by the action of heparin scavenging neutrophil lactoferrin. The use of specific tryptase inhibitors during *in vivo* dust challenge of control and heaves susceptible horses may discern pathology with a significant tryptase contribution, thereby allowing more targeted future research.

The role of not only mast cell serine proteinases, but also the many other potent mast cell mediators, in the pathogenesis of the pulmonary inflammatory response remains to be fully elucidated. However, this series of studies has illustrated mast cell participation in the pathogenesis of equine heaves.

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